·STUDIES

FROM

THE ROCKEFELLER INSTITUTE

FOR MEDICAL RESEARCH

REPRINTS

VOLUME XL

Results of the investigations conducted at The Rockefeller Institute, or elsewhere under its grants, are first reported in a variety of journals and publications. The reports are then assembled in volumes designated Studies from The Rockefeller Institute for Medical Research, of which this is Number XL. The Studies appear serially but at irregular intervals. The text of the original publications is in all respects followed in the Studies. The name, date, number, and pages of the journal in which each article originally appeared are printed above the title. To insure uniformity and simplicity of reference, plates and illustrations repeat the numbers used in the place of first publication.

CONTENTS.

iii

BIOPHYSICS.

PHYSIOLOGY AND PHARMACOLOGY.

CHEMISTRY.

EXPERIMENTAL SURGERY.

GENERAL PHYSIOLOGY.

THE HOSPITAL OF THE ROCKEFELLER INSTITUTE.

ANIMAL PATHOLOGY.

LATENT INFECTIONS WITH THE DEMONSTRATION OF SPIROCHÆTA PALLIDA IN LYMPHOID TISSUES OF THE RABBIT.

By WADE H. BROWN, M.D., and LOUISE PEARCE, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

In human syphilis there frequently comes a time during which the patient, although known to be infected, shows no obvious manifestation of an active syphilitic process. Within recent years, more exact clinical methods have shown that some of these patients are the subjects of visceral or of nervous involvement and are, therefore, cases of obscure rather than of latent infection. The work of Warthin[1] has shown further that active pathologic processes may exist where there are no clinical signs to indicate their presence and finally that spirochetes with more or less tissue alteration may be demonstrated in individuals where there was little if any evidence either of disease or of infection. The number of cases of latent syphilis, known and unknown, is probably a very considerable one and is of increasing importance to the syphilographer.

In rabbits, latency is a regular phenomenon of the infection. The primary lesions develop and heal spontaneously within a period varying from a few weeks to more than a year and if lesions occur elsewhere they pursue the same course. Within a few months from the time of inoculation therefore no manifestations of infection may be found. This phenomenon has been spoken of as spontaneous recovery and is generally regarded as such since very few cases of relapse have been reported.

Relapses do occur, however, and while no estimation of their frequency can be given, they are more or less proportionate to the length and carefulness of the observation of infected animals. Spontaneous recovery in the rabbit is frequently only temporary and lesions of some kind may recur after the lapse of a few months and occasion-

ally a year or more may intervene. During such intervals as these, the most careful examination fails to reveal the presence of a syphilitic lesion, either clinically or pathologically. These are cases of true latency or cases in which the animal, although still infected, has acquired some power of restraint upon the growth and multiplication of spirochetes and protection, for the time being, against their harmful effects.

If relapse occurs, we know that the animal was still infected but otherwise there is but one condition to indicate that infection may still exist and this is the presence of some degree of abnormality of the superficial lymph nodes, especially the popliteals. The changes noted in cases of latent infection vary from a shotty induration of small nodes to a moderate enlargment with some degree of induration.

This observation taken in connection with previous work on lymph node involvement during various stages of active infection[2] led us to undertake a small series of experiments which had two objects in view, namely, the demonstration of infection following so-called spontaneous recovery or during periods of latency, as the case might be, and the determination of the location of the spirochetes during such periods. The apparent abnormality of the popliteal nodes and the fact that in the active stages of infection spirochetes were always demonstrable in these nodes by animal inoculation suggested that the simplest method of approach to this problem was by excision of popliteal nodes and the inoculation of test animals according to methods described in a previous paper.[*2]

Experimental Demonstration of Spirochetes in the Popliteal Nodes.

The experiments to be reported were carried out on six rabbits all of which had shown well marked generalized manifestations of disease, but at the time the investigations were made four of them showed no lesions of any kind, while the other two showed slight lesions of an indifferent character in which no spirochetes could be demonstrated by dark-field examination. Three of the rabbits were infected with the Nichols strain of Spirochete pallida and three with the Zinsser-Hopkins strain.

*All operative procedures were carried out under ether anesthesia.

For purposes of orientation, the essential points in the history of the six animals may be summarized as follows:

Rabbit No. 1.—Zinsser-Hopkins strain. Chancres of both scrota, recurrent chancres, and recurrent lesions of the skin and iris (slight).

Duration of infection, four years and three months; length of latent period, six months (?).

At the time of examination, there was a small area of thickening in the left scrotum the surface of which was covered with fine scales. No spirochetes could be demonstrated by dark-field examination. The popliteal nodes were slightly enlarged and indurated. The right node was resected for inoculation. The capsule and the node were slightly fibrous.

Rabbit No. 2.—Zinsser-Hopkins strain. Double orchitis with marked skin involvement followed by pronounced lesions of the bones and slight lesions of the skin and mucous membranes.

Duration of infection, nine months; length of latent period, three months.

At the time of examination, there were no demonstrable lesions present. The popliteal nodes were small but slightly shotty. The left node was removed for inoculation. The node was small and of a pale yellow color; the capsule was thick and opaque.

Rabbit No. 3.—Zinsser-Hopkins strain. Double orchitis followed by complete atrophy of both testicles; recurrent lesions in the testicles and scrota; pronounced involvement of bones, skin, cornea and iris with slight lesions of the mucous membranes.

Duration of the infection, seven months; length of latent period, three months.

At the time of examination, no lesions could be detected. The popliteals were moderately enlarged and indurated. The left node was removed. The capsule was thick and opaque and the node itself somewhat fibrous.

Rabbit No. 4.—Nichols strain. Double orchitis with marked skin involvement. Unusually marked affection of skin, bone, mucous membranes and eyes—a case of malignant syphilis.

Duration of infection, nine months; length of latent period, three months (?).

At the time of examination, there was a slight infiltration about the margins of the right nostril and there was some discharge from the nose. Examination for spirochetes was negative. The popliteal nodes appeared to be slightly enlarged and indurated. The right node was resected for inoculation. The nodal mass was composed mostly of fibrous tissue; the parenchyma was small in amount.

Rabbit No. 5.—Nichols strain. Inoculated in the right testicle. Marked orchitis with skin involvement, metastatic lesion of the left testicle, marked cutaneous lesions and moderate infection of the bones.

Duration of infection, seven months; length of latent period, three months.

At the time of examination, no lesions were present. The popliteal nodes were moderately enlarged and indurated. The left node used for inoculation. The capsule was fibrous and thickened while the node itself was quite large, somewhat mottled and of a slightly yellow color.

Rabbit No. 6.—Nichols strain. Inoculated in the right scrotum. The scrotum and testicle were amputated forty-eight hours after inoculation. There followed a generalized disease with lesions of the left scrotum and testicle, marked bone lesions, and some lesions of the skin.

Duration of the infection, seven months; length of latent period, three months.

At the time of examination, no lesions were present. The popliteal nodes were distinctly enlarged and indurated. The right node was resected; its capsule was thickened while the node itself was enlarged and somewhat fibrous.

An emulsion was prepared from the popliteal nodes of each of these animals and 0.5 cc. was injected into the right testicle of two normal rabbits. The results of the injections are shown in Table I.

TABLE I.

Results of Inoculations from the Popliteal Nodes of Rabbits During Latent Periods of Infection.

Source animal.	Duration of infection.	Length of latent period.	Number of test animal.	Results of inoculation.	Incubation in days.
1*	51 months	6 months	1	+	38
			2	+	43
2	9 "	3 "	1	+	42
			2	+	44
3	7 "	3 "	1	+	37
			2	+	39
4*	9 "	3 "	1	+	34
			2	+	31
5	7 "	3 "	1	+	37
			2	Dead	
6	7 "	3 "	1	+	31
			2	+	31

+ Indicates infection.

* These two animals showed suggestive lesions at the time the examinations were made. They subsequently increased but no spirochetes could be demonstrated in them by dark-field examination.

The noteworthy features of these experiments are the constancy with which infection was demonstrated and the rapidity with which infection developed in all of the test animals. In these respects, the results practically coincided with those previously obtained from the inoculation of lymph node material from cases of acute or active syphilitic infection. The incubation period was, in some instances,

a few days longer, but averaged about the same in the two cases and was shorter than that usually obtained from blood inoculations except at the most active periods of infection. This was more than was to be expected.

It may be noted also that there was a degree of parallelism between the pathologic condition of the node and the length of the incubation period, which was shorter in the case of nodes, showing a medullary swelling than in those which were atrophic and fibrous.

Since the inoculations were made, four of the animals have shown no change from the condition recorded above and their period of latency has been extended by more than two months. In the case of Rabbit No. 1, the small area of infiltration in the scrotum increased in size until it formed an indurated nodule about 1 cm. in cross diameter and 0.5 cm. in thickness.

Rabbit No. 4 has also shown an increase in the lesions mentioned. Subsequent to the removal of the node for examination, there was an extension of the infiltration from the right nostril to the left and, while no examination has been made for spirochetes, the lesions which developed and are still present present the typical appearance of syphilitic lesions in this locality.

The duration of infection or of the period of latency in the group of animals reported is not unusual except in Rabbit No. 1. The infection in this animal has at all times been a mild one; only a few lesions have developed and these have been comparatively slight. Nevertheless, virulent organisms have remained alive in the animal for approximately four years and a half. This is by far the longest case of animal infection on record and indicates very conclusively that the apparent recovery which takes place in these animals is only a symptomatic one.

<div align="center">DISCUSSION.</div>

These experiments are the first to show that rabbits which have recovered from the clinical manifestations of syphilis are still infected and that they may continue to harbor virulent spirochetes even though they show no clinical or pathologic evidence of infection except for the adenopathy described.

The continued existence of virulent organisms in these animals and the absence of lesions are very signficant facts and indicate, as we believe, that some distinction may be drawn between immunologic processes which affect the growth and multiplication of the spirochetes and those which have to do with their localization and the development of lesions or the toxic effects of the organism.

Again, the constant recovery of spirochetes from the lymph nodes and the infectiousness of this material carry with them suggestions of possible seats of predilection for the spirochetes during latent periods of infection. Little is known of the ultimate location of the organisms during such phases of either the human or animal infection. The problem is difficult of approach from the human side and experiments thus far carried out on animals have not contributed to its solution.

During late stages of human syphilis, spirochetes have been demonstrated in a variety of tissues ranging from scars of the skin to the central nervous system—usually, however, in association with a definite syphilitic process. Likewise systematic examinations of the tissues of infected animals have been made by several investigators during periods of active infection, using the method of animal inoculation. In this way, spirochetes have been recovered from a variety of sources, but the signficance of the findings is rendered uncertain by the fact that at such times a septicemia exists which makes it impossible to differentiate between organisms in the blood passing through an organ and those localized in the tissues.

With the healing of the lesions, spirochetes disappear from the blood of the rabbit and numerous blood examinations made by us during latent periods of the infection (including one animal of the above group), have invariably given negative results. This source of confusion is, therefore, removed from the experiments which we have reported.

The work thus far done tends to show that a widespread distribution of spirochetes exists but it has not shown the points at which they come to rest during latent periods of infection. It is doubtful whether such points could be determined with absolute certainty by the means at our disposal, but the facts now known are sufficient to lend support to the assumption that the lymphoid tissues are the ones chiefly concerned.

Syphilographers have long recognized the existence of an affinity of Spirochete pallida for lymphoid tissues and the adenopathy of syphilis is one of its most characteristic features. In like manner, it has been shown that, in all lesions, the points at which the spirochetes tend to accumulate in greatest numbers are the perivascular lymphatics.

These same conditions are equally true for the rabbit and during active periods of infection spirochetes can always be recovered from superficial lymph nodes with greater ease and in greater number than from the blood; at times the nodes are almost as infectious as the active lesions. With the disappearance of lesions and of spirochetes from the blood, a condition develops in the rabbit which may be regarded as one of true latency. During this period, a slight adenopathy persists and spirochetes can still be recovered for months or even years from the most easily accessible masses of lymphoid tissue.

The assumption seems warranted, therefore, that while the spirochetes are widely distributed over the body during latent as well as active periods of infection, the distribution is not an indiscriminate one but that the chief reservoirs of infection are the lymphoid structures of the body whether massed as in the case of the lymph nodes or in the form of the simpler perivascular lymphatics. This points to the possibility of a wider application of our knowledge of lymphoid involvement in diagnostic and prognostic measures.

SUMMARY AND CONCLUSIONS.

Six rabbits which had recovered from generalized syphilis were used as the basis for determining whether such animals were still infected and something as to the location of the spirochetes in cases of latent infection.

One of the animals was inoculated four years and three months prior to the examination, another nine months, and the others seven months. At the time the examinations were made, all of the animals showed a suggestive adenopathy which was most evident in the popliteal nodes. In addition, two of them showed slight lesions of an indifferent character in which no spirochetes could be demonstrated by dark-field examination. The others showed no lesions. The

latent period of infection was of three months duration in five of the animals and was six months in the other.

A popliteal node was removed from each of the animals and used for a test inoculation of two normal rabbits. Infection was produced in all cases, the incubation period varying from 31 to 44 days, which is practically the same as that given by lymph node inoculations during active stages of infection and shorter than that obtained from blood inoculations except in the most active stages of infections.

From these facts, it may be concluded that rabbits which have recovered from clinical manifestations of syphilis may harbor virulent spirochetes almost indefinitely even though no further manifestations of infection should occur. Moreover, the infectivity of material from the popliteal nodes, taken in conjunction with other evidence of an affinity of spirochetes for lymphoid tissues, is interpreted as indicating that the lymphoid tissues of the body in general are probably the chief reservoirs of the virus during latent periods of syphilitic infection. From this, it is suggested that a wider application may be made of our knowledge of lymphoid involvement in the management of cases of human infection.

REFERENCES.

[1] Warthin, A. S.: Amer. Jour. Med. Sci., 1916, clii, 508.
[2] Brown and Pearce: Arch. Dermat. and Syph., 1920, ii, 470.

EXPERIMENTAL PRODUCTION OF CLINICAL TYPES OF SYPHILIS IN THE RABBIT.

BY WADE H. BROWN, M.D., AND LOUISE PEARCE, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

Syphilis in the human subject is characterized by the occurrence of a succession of lesions of various types which follow one another in a more or less orderly progression from the chancre to the terminal stages of the infection, and while to each period of the infection certain types of lesions have been assigned, the order of their appearance is not entirely fixed nor can one say just what lesions may be expected to occur in a given case.

The factors that determine these features of the disease may be numerous, and the results of their action differ so widely that diversity rather than uniformity has come to be recognized as one of the most fundamental characteristics of syphilis. The causes which underlie this diversity of clinical types are but little understood. It is of course obvious that there are two sets of factors which may contribute to this condition, namely the reactive mechanism of the host and the disease producing properties of the organisms concerned.

Within recent years, the tendency has been to lay more and more stress on the influence of the latter group of conditions. The possibilities of the existence of so-called strains of *Spirochaeta pallida* was first suggested by Noguchi[1] and later made the subject of experimental investigations by Nichols,[2] Reasoner[3] and others. More recently this idea has been emphasized by the work of Levaditi, Marie[4] and their co-workers in connection with general paresis. As yet, however, the subject of the cause of clinical variation is an open

1. Noguchi: J. Exper. Med. **15**:201, 1912.
2. Nichols: J. Exper. Med. **19**:362, 1914.
3. Reasoner: J. A. M. A. **67**:1799, 1916.
4. Levaditi and Marie: Ann. Int. Past. **33**:741, 1919. Marie, Levaditi and Banu: Compt. rend. Acad. d. Sc. **170**:1021, 1920.

9

one, and while syphilographers have recognized the fact that many peculiarities of the infection may be due to causes other than strain differences, this possibility has been more of less neglected by experimental investigators.

It is not easy to approach the problem of clinical variation by means of observation on the human subject, since such a small part of the phenomena of infection can be observed in an individual case, and neither the host nor the causative agent is subject to the necessary measure of control. In the experimental animal, however, a large part of these difficulties are removable, and it is possible to reproduce many of the phenomena of the human infection under conditions of acceptable control. Thus by local inoculation into the testicles or scrotum of the rabbit, one may obtain an infection which begins with a primary lesion and after a lapse of from six to twelve weeks is marked by the appearance of a variety of lesions in other parts of the body, together with a general lymphadenitis and at times by constitutional disturbances; there are lesions of the skin and appendages, the mucous membranes, the periosteum and bones, and of the eyes. The infection is not identical with that in man, but the analogy is sufficiently close for one to make comparisons, and, at the same time, the differences are sufficiently great to enable one to make clear-cut experimental distinctions.

Variation in the clinical course of the disease is characteristic of the animal, as it is of the human, infection, and there are, as we have said, two aspects of the problem of variation, only one of which has been dealt with from an experimental standpoint. The subject of variation is an intricate one and at this time we do not propose to consider the ultimate possibilities of either of the potential factors concerned.

It has been noted, however, that with the use of a given strain of *Spirochaeta pallida,* one may still obtain infections which exhibit decided clinical variations, not only in the type of lesions which appeared in a given group of tissues, but equally so in the groups of tissues involved, and this is of more fundamental importance. An analysis of the circumstances under which such variations occurred showed an appreciable connection between the experimental conditions employed and the type of infection produced. This was

especially noticeable in the frequency with which keratitis and iritis occurred following the use of one set of conditions and the frequency with which lesions of the periosteum and bone followed another.

Previous experiments had shown that, while generalized lesions occurred in only a small percentage of rabbits inoculated by certain methods, it was possible to modify the course of the infection to such an extent that the occurrence of generalized lesions became the rule rather than the exception.[5] This work was founded on a modified conception of the theory of inhibition. That the development of a lesion in one testicle was capable of inhibiting the development of other lesions in the rabbit was first demonstrated by Nichols[6] in connection with metastatic lesions of the testicles and applied by him in an explanation of the phenomena of relapse in human syphilis with especial reference to neurorecidives.[7]

Realizing the importance of the influence which the primary reaction exerted on the character of the infection in the rabbit, we were first able to convert a local into a general disease by diminishing or suppressing the testicular reaction. With a knowledge of what had been accomplished in this respect, it appeared that one might go a step further and, by modifying the defensive reaction in different ways, produce types of disease that would conform to alterations in the mechanism of animal resistance, the infecting organism being maintained as a constant factor in the reaction.

EXPERIMENTAL MODIFICATION OF THE INFECTION.

Experiments that have been carried out show that definite type alterations can be produced in response to the use of experimental procedures which influence the reaction to infection. The character of the changes, and the manner in which they were obtained may be illustrated by the following experiment:

Forty-four rabbits were inoculated in the testicles with the Nichols strain of *Spirochaeta pallida*, using for each testicle 0.5 c.c. of an emulsion containing numerous spirochetes, as many as four to eight to the microscopic field. The animals were divided equally into two

5. Brown and Pearce: Arch. Dermat. & Syph., 1920, 11:675.
6. Nichols, H. J.: J. Exper. Med. 14:211, 1911.
7. Nichols, H. J.: J. A. M. A. 63:466, 1914.

series, A and B.   Those of Series A were inoculated in both testicles
and those of Series B in only one testicle.   Lesions were palpable in
the testicles at from ten to fourteen days after inoculation.   Each
series was then further divided into four groups, making each group
as nearly comparable to the others of the series as possible from the
standpoint of the animals and the state of the testicular infection.
The several groups were handled as follows:

Group 1; Controls; Six Animals.—The infection in this group of animals was
permitted to progress without interference of any kind.

Group 2; Early Castration; Five Animals.—These animals were castrated four-
teen days after inoculation.   In Series A, both testicles were removed; in Series
B, only the infected testicle was removed.[8]

Group 3; Late Castration; Five Animals.—Castration was carried out as in Group
2, twenty-eight days after inoculation.

Group 4; Therapeutic Suppression of Testicular Reaction; Six Animals.—These
animals were given a single intravenous injection of arsenophenylglycyl dichloro-
m-aminophenol for the purpose of temporarily suppressing the reaction in the
testicle and of lowering the animal resistance.

All animals were kept under observation for from three to three and a half
months after inoculation.   During this time, they were examined almost daily
and full records of the progress of the infection were kept.

The purpose of some of these procedures may require a brief
explanation.   Early castration was employed as a means of aborting
the testicular reaction early in the course of the infection and thus
shifting the defensive reaction to other tissues before any considerable
degree of protection had been developed.   The use of late castration,
on the other hand, represented an attempt to permit the infection to
progress to a point at which the protection developing from the pri-
mary foci was nearly, but not quite, sufficient to prevent the occur-
rence of lesions in other parts of the body.   The success of this
attempt hinged on the judgment of the proper time at which to inter-
vene, and castration was performed as the first cycle of testicular
reaction was nearing completion.   The progress of the infection was,
of course, not uniform in all animals, and the time fixed for castration
was based on the average animal of the group; this was too early in
some cases and too late in others, but such irregularities could not
be avoided if a constant time element were to be maintained.

8. All operations were done under ether anesthesia.

The use of a therapeutic agent in this series of experiments was intended to accomplish more than a temporary restraint on the primary lesions. The effects produced by such agents are not entirely measurable by the reduction which they produce in existing lesions and, while we cannot discuss the action of therapeutic agents, it is necessary to state that in addition to any effect which may be attributable to a temporary suppression of existing lesions, therapeutic agents frequently nullify any protection that has been developed and render subsequent reactions on the part of the animal less effectual. It was for this purpose that arsenophenylglycyl dichloro-m-aminophenol was used.

The period of observation chosen was fixed with a view to including only one cycle of general reaction. Clinical relapse is prone to to occur in the animal as in the human subject and the inclusion of this class of phenomena would introduce more complex elements, which cannot be considered in this article.

<div align="center">RESULTS OBTAINED.</div>

The influence which these procedures exerted on the course and character of the infection may be learned from a comparison of the case incidence and relative severity of generalized lesions, the groups of tissues involved, the order of involvement and the time after inoculation at which the lesions appeared. For convenience, these facts have been tabulated in parallel columns in the table. The list of conditions given includes no lesions of uncertain or doubtful character. When any doubt existed, the condition was recorded as suspicious or doubtful, as the case might be, and animals showing such affections are marked with an asterisk (*).

As one examines the data recorded in the table, the first thing to be noted is that the occurrence and the severity of generalized lesions among animals of a given group was inconstant. This indicates the existence of a variable in the reaction of individual animals to a given set of conditions which must be regarded as present in all cases.

Considering the character of the infection which occurred under the several conditions employed, it will be seen that it varied from an infection in which scarcely any lesions were demonstrable (except those in the testicles), as in Group 1 A, to infections in which a vari-

Effect Produced on the Character of the Infection by Modifying the Reaction of the Host in Animals Inoculated with a Constant Dose of a Given Strain of Spirochæta Pallida.

Series A: Bilateral Inoculations.			Series B: Unilateral inoculations.		
Number and Group.	Generalized Syphilis.	Lesions in Order of Their Appearance, Time in Days.	Number and Group.	Generalized Syphilis.	Lesions in Order of Their Appearance, Time in Days.
Group 1A			**Group 1B**		
1	—*		1	—*	
2	—		2	++	S.(57, 78) E. (78)
3	—		3	—*	
4	—*		4	++	P.B.(55) E.(78,84)
5	—		5	—	
6	+	S.(57)	6	++++	S.(57,71) P.B.(63)
Group 2A			**Group 2B†**		
1	—*		1	++	P.B.(50)
2	++	S.(28) MM.(41)	2	++	MM. (41) P.B. (73, 78)
3	—		3	+	P.B.(57)
4	++	P.B.(79) S.(89)	4	++	P.B.(41,57) S.(57)
5	+++	P.B.(57,78)S. (79) E. (85)			
Group 3A			**Group 3B†**		
1	++	P.B.(57) S.(71)	1	+	MM.(63)
2	—*		2	—	
3	+++	S.(29) MM.(63) E.(80,84)	3	+++	P.B.(50) S.(50,76)
4	+	E.(85)	4	—*	
5	++	S.(50) E.(65)			
Group 4A			**Group 4B**		
1	+	P.B.(63) S.(71,78,80)	1	++	P.B.(55) S.(90)
2	+	P.B.(57)	2	+++	P.B.(55,57)
3	—*		3‡	+	P.B.(47)
4	+++	P.B.(50) S.(50,57) MM.(71)	4	++	P.B.(55) E.(97)
5	++	P.B.(41)	5	++	S.(35) P.B.(59)
6	++	P.B.(71,78)	6	++	S.(63) MM.(97)

—Negative.   +++ Well marked infection.
+Slight infection.   ++++ Extremely marked infection.
++Moderate infection.

E., eyes; MM., mucous membranes and mucocutaneous borders; P.B., periosteum and bone; S., skin.

*Animals showing suspicious lesions, not definite.

†One animal in each of these groups died before results could be determined.

‡The animal developed symptoms of acute meningitis as the first lesions were appearing and was anesthetized and killed.   Spirochetes were recovered from the cerebrospinal fluid.   There was no bacterial infection.

Figures in parentheses ( ) give the time in days in which the lesions appeared.

ety of generalized lesions occurred in practically all the animals of the group. This is the first respect in which it can be seen that a modification in the type of the infection was produced.

The next condition to be noted is that the character of the disease among the animals of a given group also varied. In Group 1 B, for example, there was one case of skin and eye infection, another of bone and eye infection and a third of skin and bone infection; or, there were two cases each of skin, bone and eye infection. A similar condition prevailed in Group 2 A but in 2 B a different type of result was obtained. Here there was an unbroken series of cases of bone syphilis, two of the four animals showing skin or mucous lesions as well.

That the occurrence of such a group of infections cannot be regarded as a coincidence is shown by the fact that when analogous conditions were employed in Groups 4 A and B the same type of infection was again produced. Further than this, a distinctly different type of infection was produced in the animals of Group 3 A by permitting the testicular reaction to progress much longer before suppression was attempted. In only one of the four animals in this group did bone lesions occur as contrasted with its occurrence in all of those of Groups 2 B and 4 A, and five of the six animals in 4 B. The infection in Group 3 A was preeminently one of skin and eyes; three of the animals showed skin lesions and three of them eye lesions and in one the eye lesions were the only lesions present.

The prominence of the latter type of infection is not fully brought out in this series of experiments, which covered only a little more than three months. This, as we realized, did not afford a proper opportunity for the development of eye lesions, which are rarely seen until toward the end of the third month and which tend to occur later when other conditions are most marked. Skin lesions showed the same tendency to late appearance under similar circumstances, and late lesions would doubtless have been more numerous had the period of observation been longer. The occurrence of these late lesions would not have altered the general type of the infection, however, but would merely have served to emphasize the conditions described.

In contrasting the manifestations of syphilis in the rabbit with those of man, attention should be called to two conditions: First,

that the disease in the rabbit is not entirely comparable either to the acquired or the congenital form of the human infection, but presents an interesting combination of the two conditions. In the second place, the most noticeable difference in the sequence of tissue involvement is the position occupied by skin and bone infections, which is the reverse of that in man (acquired syphilis). This is to be accounted for by the fact that in the rabbit the skin is a furred coat, and for the most part is less subject to infection or to the action of a host of conditions which predispose to skin infection than is the skin of man.

<center>CLINICAL TYPES OF DISEASE.</center>

The fact that it is possible to produce a scale of variations in the clinical course of syphilitic infections by modifying the reaction of the experimental animal indicates the importance of this group of factors in determining the character of the disease. It also suggests that each group of tissue reactions, or lesions, bears some relation to the reactions or lesions of other tissues and, therefore, to the course of the disease as a whole.

We shall not attempt to give a detailed statement of these relationships, but the simplest explanation which which can be offered for the results reported is one which is based on inherent tissue susceptibility and tissue reactivity.

In the first place, the occurrence or nonoccurrence of generalized lesions in the rabbit, as well as the character of these lesions, appears to depend primarily on a protective reaction which takes place at the primary foci of infection and to some extent on the relative susceptibility of other tissues. It is difficult to say what part the latter condition plays in determining the general course of the disease, but unless a given tissue furnishes suitable conditions for the growth and multiplication of the spirochetes, no very active lesions are likely to occur, nor will the reaction in such tissues contribute materially to the defensive mechanism either locally or generally.

In the second place, it is conceivable that the protection developed from a given source may not affect all tissues, or even all parts of the body, in an equal degree or at the same rate, but that it may be extended progressively from the primary lesions to successive tissue

groups, being sufficient to protect one group of tissues at a time when little or no protection is afforded to another.

According to this conception, the tissues which become involved or the lesions which develop in any given case would represent a measure of the effectiveness and the state or progress of the reaction in that animal. That such may be the case is indicated by the fact that certain groups of tissues tend to become involved in a given order; thus, bone lesions, if they occur at all, tend to follow those of the testicles or scrotum, while keratitis and iritis occur toward the end of the cycle of tissue involvement, the skin and mucous membranes occupying an intermediate position. Further than this, it has been shown that by early and radical interference it is possible to so alter the course of the disease as to cause bone lesions practically to supplant those of the testicle in the defensive reaction; conversely, by permitting the testicular reaction to progress to a given point, it is possible to confer protection on this group of structures. In this case no bone lesions occur, but the generalized disease begins with involvement of the next group of tissues in order, producing an infection which is essentially one of the skin, mucous membranes and eyes.

Going still further, it is found that while in the majority of animals inoculated in both testicles a high degree of protection seems to be conferred on other tissues of the body, in many instances the protection fails to reach the eyes; lesions of the cornea and iris appear as the only clinical manifestations of a generalized disease.

This fact, taken in conjunction with other circumstances surrounding eye infections, is of especial interest on account of a point of view which is introduced toward certain manifestations of syphilis, especially neurosyphilis. In the rabbit, eye lesions are not only terminal and frequently the only generalized conditions that occur, but they are also relatively slight and more prone to repeated relapse than any other class of lesions. In the light of the conditions reported in the foregoing, these circumstances suggest that all tissues are not equally protected by the general reaction which occurs during the early stages of a syphilitic infection and that certain tissues which fail to receive this protection, although less susceptible to injury or infection than other tissues, may be capable of only a relatively slight degree of self-protection. This is undoubtedly the

case in the experimental animal, and if similar conditions obtain in man such conditions as neurosyphilis may be explainable on this basis.

It was the peculiar chain of circumstances surrounding eye infections which first drew our attention to the possibility of establishing the existence of a definite sequence of defensive reactions in the experimental animal with its corollary of a sequence in tissue protection and the possibility of producing infections of different types by modifying the defensive reaction at its source. The type of infection produced, therefore, by the inoculation of a given strain of *Spirochaeta pallida,* may be regarded as a resultant of the operation of the several factors indicated and subject, through this cause alone, to a wide degree of variation.

SUMMARY AND CONCLUSIONS.

In the course of investigations dealing with generalized syphilis in the rabbit, it was noted that distinctly different types of disease were frequently produced by inoculations made with a given strain of *Spirochaeta pallida,* it also appeared that there was an appreciable connection between the experimental conditions employed and the type of disease which occurred.

Since it had been found possible to convert a disease which is usually localized into a generalized disease by diminishing or suppressing the primary reaction, it appeared that by modifying the reaction in different ways one might produce types of disease which would conform to alterations in the defensive mechanism. Experiments carried out on a large series of animals showed that such was the case. By various experimental procedures, different types of infection were produced, and in this way it was found that there was a definite sequence of tissue involvement or of tissue reactions which made it possible to produce infection or to confer protection on a given group of tissues according to the nature of the means employed.

From the manner in which the infection responded to modifications of the defensive reaction, the conclusion was drawn that a wide variation in the clinical type of the disease might be traceable to this one group of causes. This, of course, does not eliminate the possibilty that variation in the biologic properties of the infecting organisms may contribute to further variations in the type of the disease.

STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

Preliminary Report on Cultivation Experiments.

By PETER K. OLITSKY, M.D., and FREDERICK L. GATES, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

In a preliminary report[1] in The Journal describing the effects produced in the lungs and blood of rabbits by an agent present in the nasopharyngeal secretions of patients within the first thirty-six hours of uncomplicated epidemic influenza, we stated that during the course of these experiments we had seen "in cultures, both from the lung tissue of affected rabbits and in the filtered nasopharyngeal washings from cases of influenza, tiny bodies, almost invisible, which decolorize by Gram's method, and which stain generally with difficulty with nuclear dyes." Further, in the more complete report on transmission experiments on rabbits with these nasopharyngeal secretions,[2] it was stated that while aerobic cultures of the affected lung tissue of rabbits were negative for ordinary bacteria, except in cases of obvious contamination, the results of anaerobic cultivation were to be described in another communication.

We now desire to report our observations on the cultivable bodies to which these references were made and which were first encountered in November, 1918, in anaerobic cultures of the filtered nasopharyngeal secretions of a case of epidemic influenza.

EXPERIMENTS AND RESULTS.

The materials employed for the cultivation experiments were the filtered nasopharyngeal washings from patients in the early and late stages of uncomplicated epidemic influenza; filtered and unfiltered lung tissue from patients who had succumbed to secondary or concurrent pneumonias; filtered and unfiltered lung tissue from rabbits experimentally inoculated, and control specimens.

1. Olitsky, P. K., and Gates, F. L.: Experimental Study of the Nasopharyngeal Secretions from Influenza Patients, J. A. M. A. **74**:1497 (May 29), 1920.

2. Olitsky, P. K., and Gates, F. L.: J. Exper. Med. **33**:125 (Feb.), 1921.

In the first cultivation tests, 1 c.c. of filtrate or 0.5 cm. cube of lung tissue was added to a large piece of sterile rabbit's kidney (from 0.6 to 0.8 gm.) in a 20 by 1.5 cm. test tube. These materials were then covered with about 10 c.c. of sterile human ascitic fluid of a p_H of from 7.8 to 8.0 but not of greater alkalinity. The tube contents were then overlaid with 2.5 c.c. of sterile liquid petrolatum and the tubes were stoppered with cotton and placed in an anaerobic jar as described by McIntosh and Fildes.[3] The establishment of anaerobic conditions in the jar was indicated by the decolorization of a tube of methylene blue in broth included with the cultures. In later experiments a layer of solid petrolatum was substituted for the liquid petrolatum so that the jar could be eliminated, and similar strict anaerobiosis of the medium could be maintained.[4]

The material was incubated at 37°C. for from eight to twelve days. On about the fifth day a slight haziness was noted, usually reaching upward 1 cm. from the kidney tissue level. This faint cloud became denser, reached its maximum about the eighth day, and by that time had extended upward to about 3 cm. from the bottom of the tube. The supernatant medium developed simultaneously a very faint opalescence, at times hardly discernible. After standing two weeks, the medium became clear, owing to the settling of the cloud to the region of the kidney tissue.

Film preparations made from the material at the very bottom of the tube—the most favorable part of the culture medium for this purpose—showed on staining with ripened alkaline (Loeffler's) methylene blue numerous minute bodies, stained purple, and distinct from the precipitated granules of protein matter. These bodies, which have been observed in numerous primary cultures and in several hundred subplants for as many as seventeen generations, are from 0.15 to 0.3 micron in length and are comparable in size to the globoid bodies found in poliomyelitis. Viewed with apochromatic lenses at 3,000 diameters, they appear to be elongated, their length being about twice to three times their breadth; hence they are bacilloid rather than coccoid. They often occur discretely, sometimes in diploform and rarely in short chain formation of three or four members. In some young cultures but more often in older ones, a clumped arrangement is discernible. These clumps range in size from that of a blood platelet to that of a polymorphonuclear leukocyte. In these masses, the individual bodies are distinct. In any case, the outline of each body is regular and the tendency to pleomorphism is not marked, although in some preparations an occasional longer body is to be seen. One culture is now in its seventeenth generation, and the form and size of the bodies therein are still similar to those in the initial culture.

When few in numbers, the bodies sometimes are obscured by protein or stain precipitate and to the unaccustomed eye they are made out with difficulty. In general they stain with difficulty with the usual dyes. Giemsa's and Manson's stains dye them a lavender shade, but are unsatisfactory on account of con-

---

3. McIntosh, J., and Fildes, P.: Lancet 1:768 (April 8), 1916.
4. Gates, F. L., and Olitsky, P. K.: J. Exper. Med. 33: 51 (Jan.), 1921.

comitant precipitation. For this reason carbolthionin (Nicolle), carbolfuschsin, steaming safranin and fuchsin are unsuitable also. They are decolorized by Gram's method. The counterstain, safranin, requires steaming. The negative reaction to Gram's stain is a constant feature of old and young cultures.

Initial cultures in a semisolid medium consisting of one part of agar to two parts of human ascitic fluid usually fail to show signs of growth. But subcultures do develop in this medium with the formation of minute colonies, too small to be exactly defined in the medium; the evidence of growth is usually noted by haziness or an even clouding of the medium from below upward to within 2 to 3 cm. from the surface.

In mass cultures, such as were employed by Flexner, Noguchi and Amoss[5] for growing the globoid bodies of poliomyelitis, a turbidity appears throughout the solid agar layer after the third day, and a diffuse though slight haziness of the fluid layer after the fifth day.

On account of their size, the dark field does not offer a satisfactory method for their detection or examination, because of their similarity in size to the familiar "dancing bodies" found also in control preparations.

Although growth is obtained in a medium containing carboyhdrates and alcohols, such as dextrose, maltose, lactose, saccharose and mannitol, these substances appear not to be attacked by the bodies. Gas is not formed, nor is any odor detectable in the culture media during growth.

' The bodies are destroyed by heating at 56°C. for one-half hour and by chloroform vapor after from one to one and one-half hours' exposure. They are capable of growing in symbiosis with a number of ordinary bacteria, among which may be mentioned *B. pfeifferi*, the pneumococcus, *Streptococcus viridans*, *Streptococcus hemolyticus* and the staphylococci.

The cultivated bodies pass through V and N Berkefeld candles.

The bodies have been cultivated from the filtered nasal washings of patients in the first thirty-six hours of epidemic influenza, rarely from specimens taken in the latter stages of the disease. They were not found in filtrates of lung tissue from patients who succumbed to secondary penumonias. They were recovered in pure culture from the filtered and unfiltered lung tissue of rabbits and guinea-pigs inoculated with the filtered or unfiltered nasal washings from fresh influenza cases and from the lung tissue of rabbits injected with active material, which had been immersed in sterile 50 per cent. glycerol for nine months.

Similar cultivation of control materials, consisting of the filtered and unfiltered nasopharyngeal washings from healthy persons free from influenza, or from those suffering from coryza, lung tissue of normal rabbits, and lung tissue of rabbits suffering from respiratory affections such as "snuffles" or pneumonia uniformly failed to yield growths of the bodies.

---

5. Flexner, Simon; Noguchi, Hideyo, and Amoss, H. L.: *J. Exper.* Med. 21: 91, 1915.

The intratracheal injection in rabbits or guinea-pigs of washed mass cultures of these bodies is followed in from twenty-four to forty-eight hours by a temperature rise, conjunctivitis and leukopenia involving especially the mononuclears. This condition endures for from one to three days; thereafter the animals return to normal. If an animal is killed during this reaction, the organs are found to be normal with the exception of the lungs, which show edema and emphysema, and hemorrhages in the parenchyma and involvement of the trachea and bronchi in the process. From these lungs, the same bodies can be cultivated.

SUMMARY AND CONCLUSION.

From the filtered nasopharyngeal washings from early cases of uncomplicated epidemic influenza and from the lung tissues of experimental animals, we have cultivated minute bodies of characteristic morphology which are strictly anaerobic, are filtrable, and withstand glycerolation for a period of months.

The effects on the blood and in the lungs of rabbits and guinea-pigs injected with these bodies are similar to those produced by the filtered and unfiltered nasopharyngeal secretions from early cases of epidemic influenza.

THE PENETRATION OF NORMAL MUCOUS MEMBRANES OF THE RABBIT BY TREPONEMA PALLIDUM.

By WADE H. BROWN and LOUISE PEARCE.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

The fact may be recorded that highly virulent strains of *Treponema pallidum* are capable of penetrating some portion of the genital mucosa of normal rabbits and setting up an infection without necessarily producing the first gross lesion at the portal of entry. This fact was recently determined in 9 rabbits and with two highly virulent strains of *Treponema pallidum*.

The experimental method employed was as follows:

The sheath of the animal was drawn forward to form a pouch into which was instilled 0.05 c.c. of a testicular emulsion rich in spirochetes. About 30 seconds were allowed for the emulsion to spread before releasing the sheath. Most of the fluid then ran out and between 0.04 and 0.05 c.c. could be recovered, showing that only a thin film of the emulsion was retained. Infection was first indicated by enlargement and induration of the inguinal lymph nodes and later by the development of a general lymphadenitis with syphilitic lesions in other parts of the body.

All animals thus far inoculated by this method have become infected. In some of them, enlargement and induration of the inguinal nodes was clearly recognizable within 24 hours after the application of the emulsion. Thus far (5 weeks) only one of the animals has developed a visible lesion on either the penis or the sheaths although several of them have characteristic lesions in the testicles and scrotum.

The observations on these animals are not yet complete and the full significance of the experiments cannot be ascertained until the course of the infection has been followed much longer. Similar experiments with other mucous membranes are in progress.

NEOPLASIA IN EXPERIMENTAL SYPHILIS.

BY WADE H. BROWN AND LOUISE PEARCE.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Neoplasia as a sequel to syphilitic infection is not uncommon in man but has never been recorded in an experimental animal. Recently we have observed an atypical growth arising from the scar of an old syphilitic lesion in the scrotum of a rabbit which may prove to be a neoplasm.

The animal was inoculated in the scrotum, June 16, 1916. Small chancres developed and then underwent spontaneous regression. Several months later, there was a recurrence and the lesion in the left scrotum persisted for some time. In October, 1920, there was a slight diffuse infiltration of the left scrotum and a small nodule appeared at the site of the old chancre. Although it was known that the animal still harbored spirochetes,[1] none could be demonstrated by dark-field examination of material from the nodule and it was excised for histological examination. There was a prompt recurrence and with the growth of the second cutaneous lesion the left inguinal glands became markedly enlarged and indurated. Again no spirochetes could be demonstrated and the lesion with one of the adjacent glands was excised under ether anesthesia. Histological examination of the cutaneous nodules and gland showed a growth which presented more the appearance of a neoplasm than of a syphilitic lesion. It was composed for the most part of atypical epithelioid cells undergoing active proliferation and exhibiting marked invasive tendencies.

Meantime there was a second recurrence and extension of the skin lesion over the mid line at the pubis with enlargement and induration of the right inguinal nodes. A deterioration in the physical condition of the animal was then apparent and progressed very rapidly, culminating in emaciation, weakness, severe anemia, loss

---

[1] See account of rabbit No. 1, *Amer. Jour. Syph.*, 1921, v. 1.

of sphincter control, with some spasticity of the hind legs and the formation of trophic ulcers about the anus and sheath.   On this account, the animal was etherized.

Post-mortem examination revealed a widespread distribution of nodules identical in character with the original lesions.   These were most abundant in the liver and bone marrow but were also present in other organs such as the spleen, the lungs, and the kidneys.

Transplantation of material from an inguinal node gave a vigorous growth in the original animal and attempts to transfer the growth to other animals have apparently been successful.

EXPERIMENTAL SYPHILIS IN THE RABBIT.

VI. AFFECTIONS OF BONE, CARTILAGE, TENDONS, AND SYNOVIAL MEMBRANES.

PART 1. LESIONS OF THE SKELETAL SYSTEM.

BY WADE H. BROWN, M.D., AND LOUISE PEARCE, M D., AND WILLIAM D. WITHERBEE, M.D., ROENTGENOLOGIST.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 52 TO 60.

Syphilitic lesions of the skeletal system of the rabbit were first reported by Uhlenhuth and Mulzer (1) in 1910 under the rather indefinite term of "nose tumors." The conditions described by them were produced by intracardial and intravenous injections of massive doses of *Treponema pallidum* in young rabbits and appear to have been chiefly tumor-like swellings of the nasal mucosa and the soft tissues about the end of the nose. However, periostitis was described in one instance, and subsequent reports (2) contained illustrations of several types of the "nose tumors," which were obviously lesions of the nasal bones and cartilages.

Reasoner (3) also has mentioned the occurrence of a periosteal lesion of the nasal bones and refers to this type of affection as a late manifestation of the infection produced by certain strains of *Treponema pallidum*. None of these authors indicates the relative incidence of this class of lesions, and these are the only references to lesions of the skeletal system which have come to our notice. Apparently no instance of such a condition has been reported following a local inoculation, unless it be the case referred to by Reasoner.

In our experience, however, localized infections of the skeletal system, including the bones of the feet and legs and face, were quite common, hence it seems that the conditions have passed unrecognized and are, therefore, essentially new additions to the subject of experimental syphilis.

Among our earlier animals, there were 33, or 26 per cent, of those showing manifestations of generalized syphilis, in which lesions of the periosteum, bone, cartilage, tendons, and tendon sheaths were recognized by simple palpation and inspection. With the introduc-

tion of methods intended to increase the incidence and the severity of the generalized infection, as indicated in a previous paper (4), there was a marked increase in this particular group of conditions and the total number of cases available for study was more than doubled. In addition, the use of the radiograph in the diagnosis and study of deep seated lesions has been of considerable advantage.

The majority of the lesions seen were instances of periosteal, perichondrial, or bone involvement; only a few cases were recognized clinically in which the lesions appeared to originate in tendons, tendon sheaths, and like structures, and in a few instances affections of the joints were noted which appeared to be attributable to syphilitic infection. The parts chiefly affected were the facial and cranial bones and cartilages, and the bones, tendons, and joints of the feet, legs, and tail.

All these are lesions of hidden parts and in order to convey some idea of the nature of the processes concerned, it will be necessary to preface the clinical description of this form of experimental syphilis with a brief description of the lesions themselves.

General Character of the Lesions.

Lesions of the skeletal system proper occurred both in the periosteum and in the deeper parts of the bone or cartilage but most of the cases which came under our observation were clearly instances of primary periosteal involvement.

Periosteal Lesions.—These were of two types, the more common one being a nodular, granulomatous condition and the other a process of a more diffuse character. The first was characterized by the formation of small flattened or oval plaques, or of elevated nodules of extreme hardness distributed over the surface of the bones. Some of them spread laterally, forming masses which could barely be detected, while others became more elevated and reached a size of from a few millimeters to more than a centimeter in diameter.

Clinically, these lesions were readily distinguishable from cutaneous affections by the fact that the skin was freely movable over them and they were firmly attached to the underlying bone. When exposed to view, the earlier lesions appeared translucent and of a pale, slightly

yellow color, or presented an opalescent appearance and were surrounded by a faint areola of newly formed vessels. As the lesions developed, they became more dense and changed to a gray or yellowish gray color. Some were highly vascular and showed irregularly distributed areas of congestion and hemorrhage together with foci of necrosis. These conditions may be illustrated in general by Fig. 1, which is a photograph of an autopsy specimen showing the condition present 11 days after the lesions were first noted.

Upon section, some of the lesions were dense and fibrous, while others were more succulent or of a fleshy character. The cut surface of the larger ones frequently presented a mottled appearance due to the presence of irregularly distributed areas of necrosis and hemorrhage. In other cases, the surface was thickly stippled with minute gray or yellow points of necrosis or the entire center of the lesion was necrotic and demarcated from an outer zone of living tissue by the presence of a gray or yellowish gray line (Figs. 2 and 3).

The necrotic portion of the lesion remained firm and elastic, as a rule, but in exceptional instances or in cases of extensive necrosis, the central portion broke down into a softened or a cheesy mass (Fig. 3).

At the beginning, lesions of this type were largely confined to the periosteum, but as the process advanced, they frequently extended to the underlying bone. The changes in the bones themselves were quite variable. In general, they consisted of erosion of the outer table, or of bony caries, and finally led to an osseous overgrowth or the formation of osteophytic nodes. The destructive changes are shown in Figs. 4 and 5.

The first photograph (Fig. 4) shows the condition found at autopsy in an animal with actively progressing lesions of the facial bones. There were three groups of lesions present, the main mass occupying a position over the bridge of the nose while smaller lesions were present on both sides at a slightly higher level.

Upon turning back the flap of periosteum covering the left side of the nose (Fig. 5), the upper lesion appeared as a thickening of the periosteum corresponding with an area of erosion in the bone beneath. Over the bridge of the nose, there was a rather large granulomatous mass, most of which was firmly united with the periosteum. In the position occupied by this mass, a considerable area of the nasal bone had been completely destroyed, exposing the periosteum of the under side of the bone which was also affected. These lesions had been recognizable for only 19 days and yet the degree of destruction was quite marked.

Pathologically, the diffuse periostitis differed from the more circumscribed or nodular form chiefly in the presence of a thin layer of rather soft and friable tissue which covered a wide area of bone. During the earlier stages of the infection, little or no alteration could be detected in the contour of the part, but as the lesions increased, slight thickenings or irregularities could be made out.

On the whole, this type of process appeared to be more destructive than the circumscribed or nodular form; that is, its extension was lateral and inward rather than upward or outward. These lesions also tended to produce a chronic fibrous thickening of the periosteum such as that shown in Figs. 6 and 7. The thickening of the periosteum is here most apparent near the end of the nose where a bulbous enlargement has been produced. This was due in part to periosteal thickening and in part to a thickening of the bone and nasal cartilages (Fig. 7).

With bones such as the nasals, periosteal lesions developed from the under side of the bone as well as from the outer side. This is shown in a low power magnification of a cross-section of the nasal bone reproduced in Fig. 8.

Perichondrial lesions, especially those about the face, presented essentially the same characteristics as those of the periosteum. During periods of active growth, spirochetes could always be demonstrated in these as well as other bone lesions to be described, and were usually present in very large numbers.

Lesions in the Bone and Marrow Cavities.—In addition to primary periosteal involvement with subsequent extension to the bone, a number of lesions were seen which originated within the bone or marrow cavities. This class of infections, including what might be designated as osteitis, osteomyelitis, and epiphysitis or osteochondritis, is still somewhat obscure owing to the difficulty in detecting the lesions at an early stage of their development.

Clinically, little could be told of these affections by ordinary methods of examination until they had reached a fairly advanced stage. Necrosis of such bones as the nasals could then be detected by a crackling or giving of the bone beneath the palpating finger, and as the process extended to the outer surface of the bone, a deformity was produced which in most instances presented the same picture as that

of a primary periostitis.   Lesions at the epiphyses of the long bones could also be detected in some instances by the presence of a swelling at the epiphyseal line, but they were difficult to distinguish from periosteal lesions which showed a marked tendency to localize at the same points.

By the use of x-rays, some early lesions were detected and their development was followed, but, as a rule, infection of the deeper parts of the bone was not discovered until the picture had been complicated by necrosis with dissolution of the bone or by pathological fracture.   There are, therefore, two groups of conditions to be considered—one including lesions of obvious syphilitic origin and the other affections of a more obscure character.

By radiographs and by pathological examination, lesions were found in both the long and the flat bones.   These appeared as focalized processes composed of a pale, translucent, and almost gelatinous material which spread out in the marrow cavities to a greater or less extent and invaded the substance of the bone.   A lesion of this type is shown in Fig. 9 which is taken from the so called fifth metatarsal. As may be seen, there were in this case both periosteal and endosteal lesions as well as focal lesions within the bone itself.   That the periosteal and endosteal lesions were not parts of the same process could be determined by differences in their histology, but such distinctions could not be made with lesions within the bone.

The internal lesions appeared to arise chiefly from the membrane lining the marrow cavity (endosteum) or in the loose perivascular tissues surrounding the larger vessels.   In the long bones, they also showed a predilection for lines of epiphyseal union as shown in Fig. 10.   This represents a fully developed lesion which originated in the epiphysis and subsequently spread to other parts of the bone.

Radiographs of the early lesions showed mainly a rarefication in the bone and a loss of the finer details of structure.   These changes may be seen in Fig. 11, which shows two very distinct areas of involvement.   One of these is in the shaft of the fifth metatarsal of the right foot (*a*) and the other at the proximal end of the corresponding bone of the left foot (*b*).   The deformity of the right tarsus and their regularity seen in the calcaneus (*c*) were the result of an older lesion of a type to be described later.

This class of infections naturally gave rise to pathological altera-
tions in the bone, which were characterized by necrosis with a more
or less gradual disintegration of the bone or by increased fragility
(when necrosis was more rapid) and the occurrence of pathological
fracture or epiphyseal separation.

With these facts in mind, attention may be called to another group
of bone lesions whose early stages are very difficult of recognition.
These include chiefly cases of necrosis, fracture, and epiphyseal sepa-
ration of the calcaneus with, occasionally, similar lesions in other bones,
and may be illustrated by the affections of the tarsus shown in Figs.
12 to 14.

The history of these affections was much the same in all cases;
namely, the sudden development of lameness, with a marked edema-
tous swelling of the entire tarsus together with some tenderness and
the presence of a crepitus. The first cases which were noticed were
regarded as traumatic injuries and no particular attention was paid
to them until it was found that similar conditions occurred as a result
of obvious syphilitic infection.

A more careful investigation was then attempted and the nature of
the injury to the bone determined by both radiographic and pathologi-
cal examination. Spirochetes could not be found in fluid aspirated
from these cases[1] and the condition was complicated by the reaction
incident to the sudden disruption of the bone.

From an examination of serial radiographs of several of these animals, abnor-
malities of the bones could be made out in some which antedated the occurrence
of the lesion in question. In Fig. 15, a peculiar defect is seen in the calcaneus
of the right foot. 19 days later, this bone gave way in the middle and anterior
portions, permitting the talus to sink downward as shown in Fig. 16, the details
of the bony structures being masked at this time by an effusion into the tissues.
There was also a dropping of the arch.

A second case is shown in Figs. 17 and 18. The first point to be noted is the
dissimilarity of the posterior ends of the calcanei. The left bone shows a narrow-
ing of the neck which extends well below the level of the tuberosity, while on the
right, there is even some fullness in this region. This bone shows a band of lighter

---

[1] The demonstration of spirochetes in such cases by animal inoculation would
be of no value, since the animals were known to have a generalized infection
capable of transmission by blood inoculation.

shadow in the position of the epiphyseal line. 8 days later (Fig. 18) there was an epiphyseal separation, or fracture, which followed this line quite closely. Other examples of a similar character were also seen.

A significant feature of this class of lesions is that among the large number of animals, infected and uninfected, which were handled during the course of this work, with one possible exception, lesions of this type occurred only in animals with obvious syphilitic lesions of other bones (note metatarsals in Figs. 11 and 12). It may be mentioned also that definite periosteal lesions of the anterior end of the calcaneus have been noted, and while no gross destruction of bone was detected in these instances, all the obscure cases of necrosis such as that in Fig. 12 have occurred in exactly the same position as that occupied by the periosteal lesions.

Histologically, however, the extent of bone involvement always proved to be greater than the gross appearance would indicate, and bones which appeared to be very little affected in the gross frequently proved upon histological examination to be honeycombed by the syphilitic process. Further description of these lesions will be found below in the section on the histological changes observed in various types of bone lesions.

Lesions of Tendons and Tendon Sheaths.—There is no record of the occurrence of syphilitic lesions of the tendons or tendon sheaths of the rabbit so far as we are aware, and the number observed by us was comparatively small.

The typical lesion in cases of primary tendon involvement was a circumscribed granulomatous process of essentially the same character as that of the periosteum, and with a few exceptions the lesions were located in the tendo achillis or its sheath. The exceptions noted were small multiple lesions involving the tendons on the dorsum of the carpus and front feet and the dorsum of the hind feet. Some of these were not recognized until the animal came to autopsy, and it is possible that other cases of a similar character might have escaped our notice.

Secondary involvement of tendons and ligaments as a result of direct extension of lesions originating elsewhere was of comparatively frequent occurrence. This was not unusual with periosteal lesions

about the carpus and tarsus and the small bones of the feet, and with cutaneous lesions of the tail and lateral surfaces of the hind feet. Fig. 2 furnishes a good illustration of this type of condition.

Diffuse Exudative Reactions Associated with Lesions of the Bones.

In addition to the lesions described, an acute exudative reaction affecting the parts immediately surrounding a focus of bone affection may be referred to briefly. This reaction rarely occurred except with lesions of the tarsus or of the small bones of the feet. In cases of infection of the metatarsals and the phalanges, there was frequently an exudation into the surrounding tissues which was composed chiefly of serum with a few leucocytes and polyblasts. Such reactions occurred most often where there was a considerable degree of bone destruction.

Similar conditions have already been referred to in connection with lesions of the tarsal bones. In addition, there were a few instances in which the reaction occurred where no bone lesion was demonstrable. The inciting factor in these cases is not known, but the subsequent history of the condition, with the development of a diffuse fibrosis and fixation of the affected parts, suggests the possibility of an involvement of synovial membranes of tendon sheaths and joint cavities. Histologically, it has been shown that the *pallidum* infection may become localized in such structures, but whether this takes place by direct extension from other lesions or as an independent focus of infection is as yet undetermined.

Histology.

Histologically, the details of the pathological process concerned in syphilitic infections of the bones are too numerous and too complex to be described in the present connection. As a means of orientation, however, it seems well to refer briefly to certain features of the lesions found during active stages of the infection. Three fairly well defined groups of conditions can be recognized: (1) a granulomatous process which is not unlike that of other syphilitic lesions; (2) a condition which is characterized chiefly by absorption or by necrosis and disintegration of bone; and (3) a lesion which combines the processes

of granulomatous proliferation, absorption, and necrosis with osseous overgrowth.   This last condition will not be considered at the present time.

The granulomatous lesions usually seen in cases of bone syphilis may arise from any part of the bone and present certain structural peculiarities.   In general, they are composed of a matrix of newly formed connective tissue with a network of capillary vessels; there are the usual polyblastic infiltration and a marked tendency on the part of these cells to assume a perivascular arrangement.   Polynuclear giant cells are also present and are frequently very numerous, especially where marked bone destruction occurs.   The picture presented in these cases is of the type of an active foreign body reaction or may even suggest that of the so called giant cell osteosarcoma.

When the lesion arises from the periosteum, one frequently finds an interesting structural arrangement of the elements which participate in the reaction (Figs. 19 and 20).   There are two or three distinct layers which correspond roughly with structural divisions of the periosteum.   There is first an outer cellular layer which is composed of loosely arranged connective tissue cells and polyblasts occupying the position of the loose areolar tissue covering the outer surface of the periosteum (Figs. 19 and 20).   Then comes a denser layer of fibroblasts which contains numerous focal and perivascular accumulations of round cells and corresponds to the dense fibrous layer of the periosteum.   Finally, either as a part of this second stratum or as a distinct layer in itself, there is a zone which corresponds with the osteogenetic layer of the periosteum.   At first, this is composed of a row of giant cells (osteoclasts) whose processes dip downward into the bone, and a small amount of newly formed connective tissue infiltrated with polyblastic cells.   This is the osteoclastic layer of the lesion and its distinctive elements, the osteoclasts, are derived from the osteoblasts of the periosteum.

The composition of periosteal lesions differs very decidedly in different classes of bones.   Thus, in bones which are preformed in membrane, such as the nasal bones, a two layered structure prevails (Fig. 19).   This is composed of the loose outer layer and a highly developed third or osteoclastic layer, while the second or fibrous layer is usually absent or but slightly developed.   The lesions of the long bones

(cartilage bones), on the other hand, show three layers, the most highly developed of which is the middle or fibrous layer.   In addition, the osteoclastic layer is more fibrous and shows relatively few giant cells.   These facts are of importance in interpreting the pathological alterations produced in different classes of bones.

The growth of some periosteal lesions is marked by an increase in the thickness of the outer layers, of others by a peripheral extension of all three layers, or by a downward growth of the inner layer.   In the first instance, the condition produced is a prominent periosteal nodule, or granuloma, in the second a diffuse periostitis, and in the third marked destruction of the underlying bone.   It is in instances of the last group that the osteoclastic layer of the lesion becomes so highly developed; in membrane bones, the greater part of the lesion in such cases presents the picture of the giant cell granuloma, but this is not true of bones which are preformed in cartilage.

Lesions arising within the bone show essentially the same composition as those from the periosteum, except for their structural arrangement, and are, therefore, difficult to distinguish from them.   Endosteal affections, on the other hand, are almost devoid of fixed connective tissue elements.   They are composed of a loosely arranged stroma which is thickly infiltrated with polyblasts and contains a network of capillary vessels.   The lesion is, therefore, more of an infiltrative than of a granulomatous process.

The alterations observed in the bone itself were quite variable.   In some instances, the bone presented a practically normal appearance except for the presence of irregularities or a honeycombing of the bone due to gradual absorption, and in some, even small fragments of bone showed a well preserved architecture (Fig. 21).   Frequently, however, there were mass necrosis and rapid disintegration of bone extending over wide areas.   The picture presented in these cases (Fig. 22) was very striking and consisted of necrosis of bone corpuscles and reduction of the ground substance to a thin and ragged framework of granular or fibrillated material which stained very faintly if at all.   The impression created was that of large masses of bone undergoing solution, and various stages in the process could be traced down to the last shadowy framework of fibers and granules (Fig. 22).

Emphasis may be placed upon these processes of bone destruction on account of the occurrence of similar changes in portions of the bone where little or no syphilitic reaction of the generally accepted type could be made out.   This was especially noticeable in the lesions of the calcaneus described above.   There were two forms of alteration to be noted.   In one group of cases, the alteration consisted of a simple bone absorption with reduction in the density and the volume of the bone; in a second, there were necrosis and disintegration.

The first group developed by widening out of the lacunæ and of the perivascular spaces, which became filled with a loose and rather cellular connective tissue.   The compact portion of long bones such as metatarsals and phalanges thus tended to become somewhat cancellous in structure, while the cancellous portion of the bone was distinctly reduced in amount.   As a result of these changes, there were a reduction in both the density and the thickness of the bones and a marked enlargement of the marrow cavity.   Normally, these bones contain some cellular marrow, but in affections of the type described, there were not only bone absorption but a conversion of a cellular (hemopoietic) to a fatty marrow which, in some cases, was complete.

Mass necrosis of portions of the bone which were apparently not directly involved by the granulomatous lesions needs no further description than that given above.

The most obvious explanation of these conditions is one based upon disturbed nutrition.   There is little doubt that the primary seat of injury in all syphilitic processes is the endothelial cell, and while those in the immediate vicinity of active syphilitic lesions suffer more than do others, it appears that the effects of the toxic products which are elaborated may reach every such element of the body.   Thus, while the smaller vessels in the immediate vicinity of a lesion are most affected, it would seem practically certain that in pronounced cases of syphilitic infection, the entire capillary mechanism is subjected to injury the degree of which varies with the individual case.

If this is applied to the bone lesions described, both the absorption type of phenomenon and the mass necrosis might be accounted for upon the basis of a reduction in the blood supply due to capillary injury in the first case and to a more extensive vascular injury in the second.   It would be going too far to assume, however, that other

factors had no part in the production of these lesions, but the reduction of blood supply due to vascular injury appears to be the simplest and most immediate explanation which can be offered.

The fact that rarefication and necrosis of bone are not necessarily parallel with the size or extent of the granulomatous lesion but may occur even in the absence of any marked granulomatous reaction is of considerable importance, and the obscure cases of necrosis and fracture of the calcaneus described above were largely due to this type of bone lesion.

Histologically, small granulomatous lesions which resemble those of the periosteum or endosteum in all essential respects have been found in the synovial membranes of joint cavities, notably those of the tarsus and the metatarsophalangeal joints. In these cases, there was infection of neighboring parts, and involvement of the joints appeared to have taken place by extension from adjacent foci of infection. While it appears probable, therefore, that primary localization in the joint may occur, this has not as yet been demonstrated.

Gross Alterations in the Bones.

Frequent reference has been made above to various types of bone injury resulting from infection of the periosteum or of the bone itself. Before concluding the description of this group of lesions, it seems well, however, to give a more definite statement as to the gross alterations which are produced.

Nasal Bones and Splints.—The most marked and characteristic lesions were those of the nasal bones and splints. The effects produced may be illustrated by the series of photographs reproduced in Figs. 23 to 30. These photographs were taken from dried skulls and are intended to show alterations produced by focal and diffuse lesions during active stages of the infection as well as the results of repair.

Focal lesions of the nasal bones, as shown in Figs. 23 to 26, may produce anything from the slightest surface erosion or roughening to a complete and sharply circumscribed defect in the bone. All of the group here shown were taken toward the end of the infection and may be regarded, therefore, as representing the maximum of bone destruction produced in each instance. In Fig. 23, there was only a surface erosion, while in the second animal of the series (Figs. 24 and 25) the

necrosis extended much deeper, forming a partial defect in the bone. This defect was easily recognizable during life, and at autopsy it was found that there was little left of the bone except a thin layer of spongy necrotic fragments loosely attached to the underlying membranes. The effect produced in this case might be compared to the saddle-nose deformity of man.

The lesion involving the upper right nasal and the maxillary process of the frontal bone in Fig. 26 shows complete necrosis and removal of the bone, resulting in the formation of a small but very sharply circumscribed defect. It should be stated that the clean-cut appearance presented by this lesion was probably due to the fact that it was of internal rather than external origin. There was a very slight external growth in this case, and when this first appeared, necrosis of the bone had already taken place.

The conditions represented in Figs. 27 and 28 differ from those preceding only in that they represent processes of a more diffuse character, which, as has been explained, are usually more destructive in their effects.

Figs. 29 and 30 represent processes of repair. The lesion in Fig. 29 was of the same type as that in Figs. 27 and 28, but led to complete necrosis of a large part of both nasal bones. The condition here shown is the regeneration effected during a period of about 7 weeks. The bones were considerably thickened throughout; in the upper portion, regeneration was very imperfect, and while the bone towards the end of the nose was of a decidedly spongy character, it was much more nearly normal than that above and the probability is that in time the entire area would have assumed this appearance.

This case illustrates a remarkable tendency on the part of the rabbit to an obliteration of the marks of syphilitic lesions in the bones. Small erosions and defects of the nasal bones are usually repaired so as to leave little if any evidence of the previous injury, and the permanent alterations produced by the most destructive lesions are much less than one might expect. This evidence usually consists of a thickened and adherent periosteum, slight irregularities in the bone, surface roughening and thickening of the bone (*cf.* Figs. 6 and 7), and the presence of slight depressions or bony prominences. The last conditions are occasionally fairly well marked, as indicated by the node on the nasal splint in Fig. 30.

Anomalous Conditions of the Parietals and Occipitals.—Before leaving this phase of bone syphilis, attention may be called to the condition of the parietal and occipital bones shown in Fig. 31. The cause of this condition is not known, but, as may be seen, there is a decided thickening of the bones which are very porous or spongy in character.

Various degrees of this condition have been observed at autopsy in a number of animals.   Most of them were instances of long standing syphilitic infection; some animals were in excellent physical condition, while others showed obvious signs of malnutrition for which no cause could be found.   In some, the bones contained an abundant red marrow; in others, the marrow was more fatty in character.

It is, of course, well known that similar changes in these bones may be produced through the development of an anemia, and there is at present no reason to regard the processes here shown as the result of a local infection.   The circumstances suggest, however, that they may be produced in response to a systemic condition resulting from the syphilitic infection.

Ulna, Radius, and Tibiofibular.—Many of the long bones such as the ulna, the radius, and the tibiofibular show little alteration in appearance at any stage of the infection.   Usually there was no more than a slight roughening of the surface of the bone with either an increase or a decrease in thickness.   These areas also showed a honeycombing of the bone, but the apparent alteration was always less than that shown by microscopic examination.

The tendency was also towards a rapid restoration to normal, and bones examined a few months after the lesion had resolved showed little if any abnormality.

Metatarsals and Phalanges.—Gross changes in the metatarsals and the phalanges were more marked.   During the active stages of the infection, there were usually surface erosion, necrosis, and disintegration, epiphyseal separation, or fracture.   Less often, osseous overgrowth or the laying down of considerable masses of soft, spongy bone occurred, while the lesion was still actively progressing (Fig. 10).

As the infection subsided and the injury to the bone was repaired, various abnormalities were observed.   These consisted of changes in the form of the bone, surface roughening, and honeycombing, together with irregular reductions or increases in thickness such as were illustrated for the nasal bones.   Many of these changes appeared to be of a semipermanent character.   The abnormality diminished with time, but in a few animals which were held for observation, marks of the previous injury were still recognizable several months after the infection had subsided.

Tarsus.—Deformities of the tarsus following necrosis, fracture, or epiphyseal separation of the calcaneus were always marked. These conditions invariably led to excessive bone formation. Necrosis and disintegration of the calcaneus were followed by the formation of an irregular granular mass of bone such as that shown in Fig. 32. The union of a fracture or of an epiphyseal separation, on the other hand, was accomplished by the laying down of an excessive amount of callus and the formation of a large mass of spongy bone (Fig. 33). These conditions were also of a more or less permanent character. The absorption of callus and the reestablishment of compact bone took place slowly—more slowly than after ordinary trauma.

SUMMARY.

From a study of a series of rabbits inoculated with two old strains of *Treponema pallidum*, it was found that localized infection of bones and tendons was of frequent occurrence and led to the formation of a variety of lesions.

The bones usually involved were those of the face and the feet and legs. Most often the lesions arose from the periosteum but developed also within the bone or marrow cavities and at lines of epiphyseal union.

Grossly, the periosteal lesions were of two types—one being a circumscribed, indurated, and nodular mass and the other a process of a more diffuse character. Histologically, the lesions presented the typical appearance of syphilitic granulomata composed of more or less distinct layers which corresponded roughly with structural divisions of the periosteum. The composition of lesions of membrane and of cartilage bones differed somewhat in this respect, especially in the development of an osteoclastic layer. Invasion of the bone with absorption and necrosis were constant features of periosteal affections and were most marked in the case of the facial bones and the small bones of the feet.   .

Lesions in the bone and marrow cavities were detected chiefly by radiographs or by the occurrence of bone destruction in the absence of periosteal involvement. They were characterized by a loss of structural detail in the bone, rarefication, increased fragility, necrosis,

pathological fracture, and epiphyseal separation associated with more or less granulomatous reaction.   Histologically, the bone lesions presented essentially the same picture as those of the periosteum, while the lesions which arose from the marrow cavities were composed chiefly of polyblastic infiltrations.   In this group of affections, the most important were those which developed at the epiphyses.

The destructive effects produced by all classes of lesions varied from a slight surface erosion or rarefication to extensive necrosis resulting in the formation of bony defects or in disintegration or fracture of the bone.   These conditions differed very decidedly with the particular bones involved.

Of especial importance in this connection was the occurrence of a peculiar form of mass necrosis which at times resulted in the destruction of considerable areas of bone even in parts where the granulomatous type of lesion was comparatively slight.   The most characteristic injuries were the saddle-nose deformities and the epiphyseal separation in the small bones of the tarsus and hind feet.

The marks of permanent injury were, on the whole, comparatively slight, but they also differed both with the degree of the original injury and with the bone affected.

Granulomatous lesions of tendons or tendon sheaths were occasionally seen, and in a few instances, lesions of synovial cavities were demonstrated microscopically.

BIBLIOGRAPHY.

1. Uhlenhuth and Mulzer, P., *Arb. k. Gsndhtsamte*, 1910, xxxiv, 222.
2. Uhlenhuth, P., and Mulzer, P., *Arb. k. Gsndhtsamte*, 1913, xliv, 307; Atlas der experimentellen Kaninchensyphilis, Berlin, 1914.
3. Reasoner, M. A., *J. Am. Med. Assn.*, 1916, lxvii, 1799.
4. Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1920, xxxii, 445.

EXPLANATION OF PLATES.

The photographs and radiographs represent the objects at their natural size. None of the illustrations has been retouched. Statements of time, unless otherwise indicated, are estimated from the date of inoculation.

PLATE 52.

FIGS. 1 to 7. Nodular and diffuse periostitis.

FIG. 1. 74 days. Typical periosteal granulomata of the nasal bones, growth tending to be upward rather than inward.

FIG. 2. 123 days. Periosteal granuloma on the shaft of the fifth metatarsal and involving the tendons of the dorsum of the foot. The cut surface shows irregular miliary areas of necrosis.

FIG. 3. 137 days. Periosteal granulomata of the tibiofibular, the fifth metatarsal, and the phalanges. The lesion of the tibiofibular shows a central area of necrosis with softening and a mottling due to congestion and hemorrhage.

FIG. 4. 84 days. Multiple periosteal lesions of the nasal bones.

FIG. 5. The same specimen with the periosteum dissected back. The lesion on the upper part of the nasal bone is of slight extent and shows only a surface erosion of the bone; that over the bridge of the nose is composed chiefly of an osteoclastic growth, and there are complete necrosis and absorption of the bone in this area.

FIG. 6. 2 years and 5 months. Chronic fibrous periostitis of the nasal bones of a diffuse type. The main lesions appear as bulbous masses at the end of the nose.

FIG. 7. The same preparation with the periosteum removed from the left side. There is an irregular thickening of the bone which is most evident in the lower third, and the periosteum was firmly adherent at many points. Note also the irregularity of the end of the nasal bone.

PLATE 53.

FIGS. 8 and 9. Photomicrographs of periosteal and endosteal granulomata.

FIG. 8. 48 days. Cross-section of nasal bone showing periosteal and perichondrial lesion arising from the inner side of the bone and working its way outward. The external coverings of the bone are normal. × 95.

FIG. 9. 89 days. Proximal end of the fifth metatarsal. The photograph shows a combination of lesions, the chief one being a granulomatous nodule in the marrow cavity; there is a distinct widening of this part of the cavity. Lesions are also seen in the bone and on both periosteal surfaces. × 30.

PLATE 54.

FIG. 10. 89 days. An epiphyseal lesion of the distal end of the fifth metatarsal. There has been a separation of the epiphysis which apparently took place in the directions indicated by the arrows. At the time represented in the photograph,

this lesion was in process of repair but there was an active syphilitic granuloma in the epiphysis. A periostitis is also seen to be present and there is an extension of the infection to the synovial membranes lining the joint cavity. × 30.

PLATE 55.

FIGS. 11 to 14. Radiographs of lesions developing within the bones.

FIG. 11. 119 days. The rarefication seen at *a* and *b* was due to osteitis of the shaft and proximal end of the metatarsals. The deformity of the tarsus at *c* was due to necrosis of the calcaneus.

FIG. 12. 163 days. Necrosis of the anterior end of the right calcaneus (marked by arrow). There were lesions also at the proximal ends of both lateral metatarsals (marked by arrows).

FIG. 13. 77 days. Pathological fracture of the middle of the calcaneus with effusion into the tissues.

FIG. 14. 73 days. Epiphyseal separation of the right calcaneus. The left calcaneus also shows some degree of abnormality which is indicated chiefly by the thickening of the upper surface of the bone toward its posterior extremity. Normally the shadow of this portion of the bone forms a straight line (*cf.* other radiographs).

PLATE 56.

FIGS. 15 to 18. Radiographs showing lesions of the bone.

FIGS. 15 and 16. Successive stages in the development of a lesion of the right calcaneus. Fig. 15 (85 days) shows a peculiar defect in the lower portion of the calcaneus which is marked by an arrow. The left calcaneus is normal. Fig. 16, which was taken 19 days later, shows an outspoken deformity of the right calcaneus. This was due in part to a dropping of the arch from involvement of the ligaments and in part to necrosis of the calcaneus, the talus becoming embedded in its substance. Note the relative position of the talus in Figs. 15 and 16.

FIGS. 17 and 18. Stages in the development of an epiphyseal separation. In Fig. 17 (77 days) there is a broad band of rarefication at the epiphyseal line of the right calcaneus (marked by arrow) and a suggestion of a separation is seen at the top of this line. The posterior end of the left calcaneus also shows a narrowing of the neck which is abnormal. Fig. 18, taken 8 days later, shows a separation of the epiphysis of the right calcaneus which followed the line indicated in Fig. 17.

PLATE 57.

FIGS. 19 and 20. Photomicrographs showing characteristic periosteal lesions of membrane and cartilage bone.

FIG. 19. 60 days. Periostitis of the nasal bone showing an outer cellular layer and an inner osteoclastic layer. The cellular layer is composed chiefly of fibroblasts with a moderate degree of polyblastic infiltration. It is also quite vascular. The osteoclastic layer is composed of fibroblasts and polyblasts together with a large number of osteoclastic cells. × 95.

FIG. 20. 77 days. Periostitis of the metatarsal. The lesion shows three distinct layers which present much the same general appearance. The two outer layers show a considerable degree of polyblastic infiltration; they are more vascular than the inner layer and the vessels show well marked endarteritis. The osteoclastic layer is but slightly developed; it is largely fibrous in character and contains very few osteoclasts. × 95.

PLATE 58.

FIGS. 21 and 22. Photomicrographs, taken from the nasal bone, showing different types of bone destruction.

FIG. 21. 60 days. Necrosis and absorption of bone accomplished chiefly by surface action. Note the wave lines in the bone, the preservation of architecture, and the survival of bone corpuscles. × 135.

FIG. 22. 100 days. Mass necrosis with rapid disintegration of bone—a type of lesion frequently observed in cases of marked bone destruction as it occurs in an acutely progressive process. × 135.

PLATE 59.

FIGS. 23 to 26. Photographs of dried preparations showing the maximum bone injury produced by various types of lesions.

FIG 23. 121 days. Surface erosion of the nasal bones produced by a large periosteal granuloma, the growth of which was mostly in the outer layers.

FIG. 24. 118 days. Necrosis of the nasal bones with the production of saddle-nose deformity due to an invasive periosteal lesion.

FIG. 25. The same specimen viewed from the side.

FIG. 26. 124 days. A defect of the right nasal bone produced by a lesion arising from the inner side of the bone. There was only a suggestion of periosteal thickening in this area.

PLATE 60.

FIGS. 27 to 33. Bone lesions and the deformities produced in the process of repair.

FIG. 27. 123 days. A widespread necrosis of the nasal bones resulting from a diffuse periostitis which produced only a slight palpable thickening over the surface of the bone.

FIG. 28. 137 days. Similar lesion viewed from the side. Note especially the destruction of the margin of the bone, the involvement of the lateral splints, and extension of the lesion to the premaxilla.

FIG. 29. 121 days. An imperfect regeneration of the nasal bones following almost complete necrosis of the areas involved. In the upper portion of the right nasal, the bone is very irregular, while below it is comparatively smooth but considerably thickened, and a defect is still present in this part of the bone.

FIG. 30. 130 days.   An osteophytic node at the point of union of the frontal process of the premaxilla and the maxillary process of the frontal bone (nasal splints), illustrating a tendency of syphilitic lesions to become localized at points of bony union.

FIG. 31. 342 days.   A thickened spongy condition of the parietal and occipital bones occurring late in the course of a generalized infection.   The exact cause of the condition is not known.

FIG. 32. 126 days.   A deformity of the tarsus resulting from necrosis of the calcaneus.   This bone is considerably thickened and extremely irregular.

FIG. 33. 126 days.   A deformity produced by an epiphyseal separation of the calcaneus.   The original lesion healed with the formation of a large mass of callus about the posterior end of the calcaneus and the separated epiphysis.
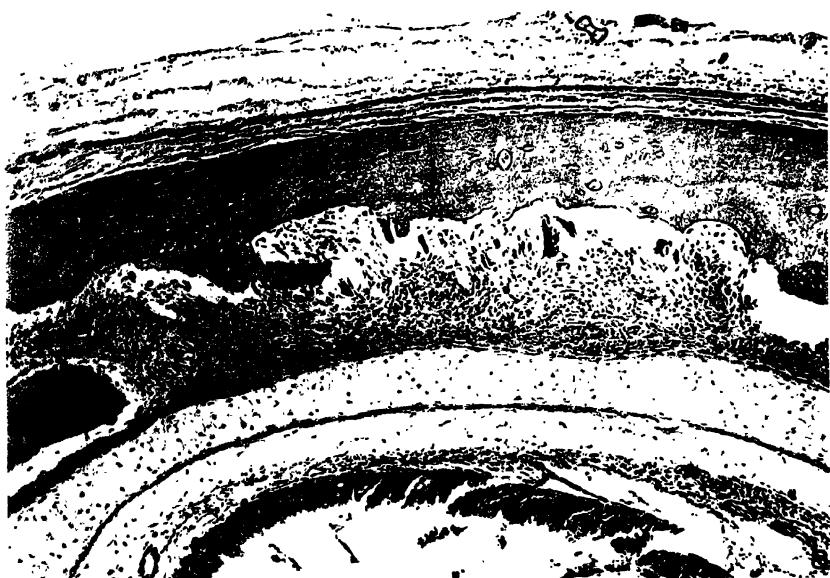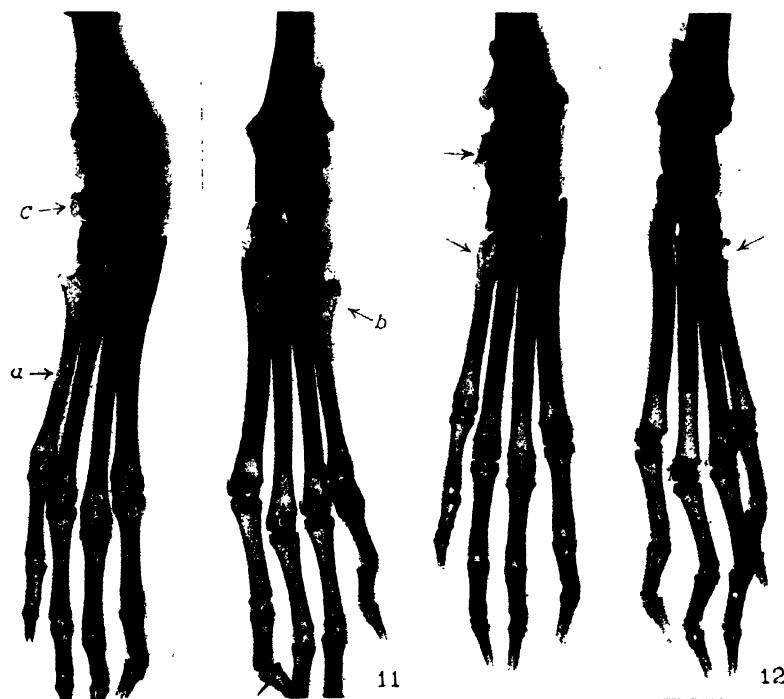
9

Right 15

Right 16

Right 17

Right 18
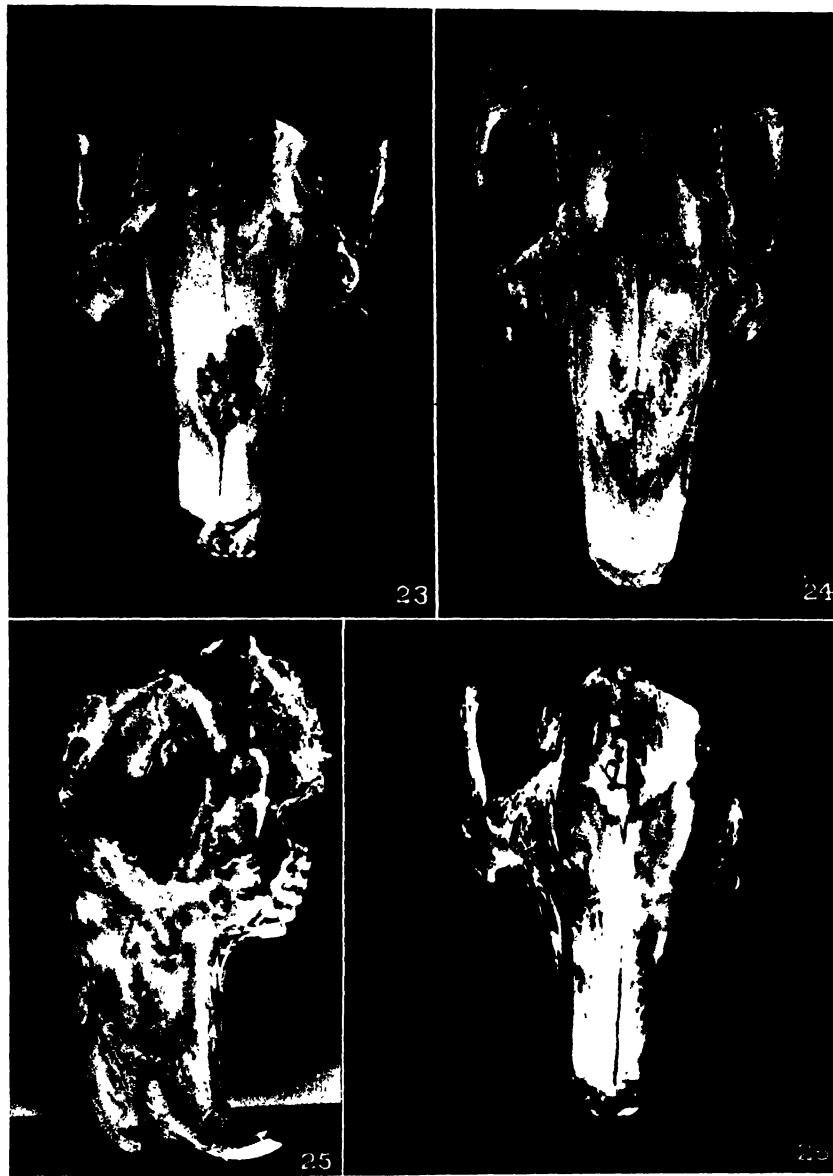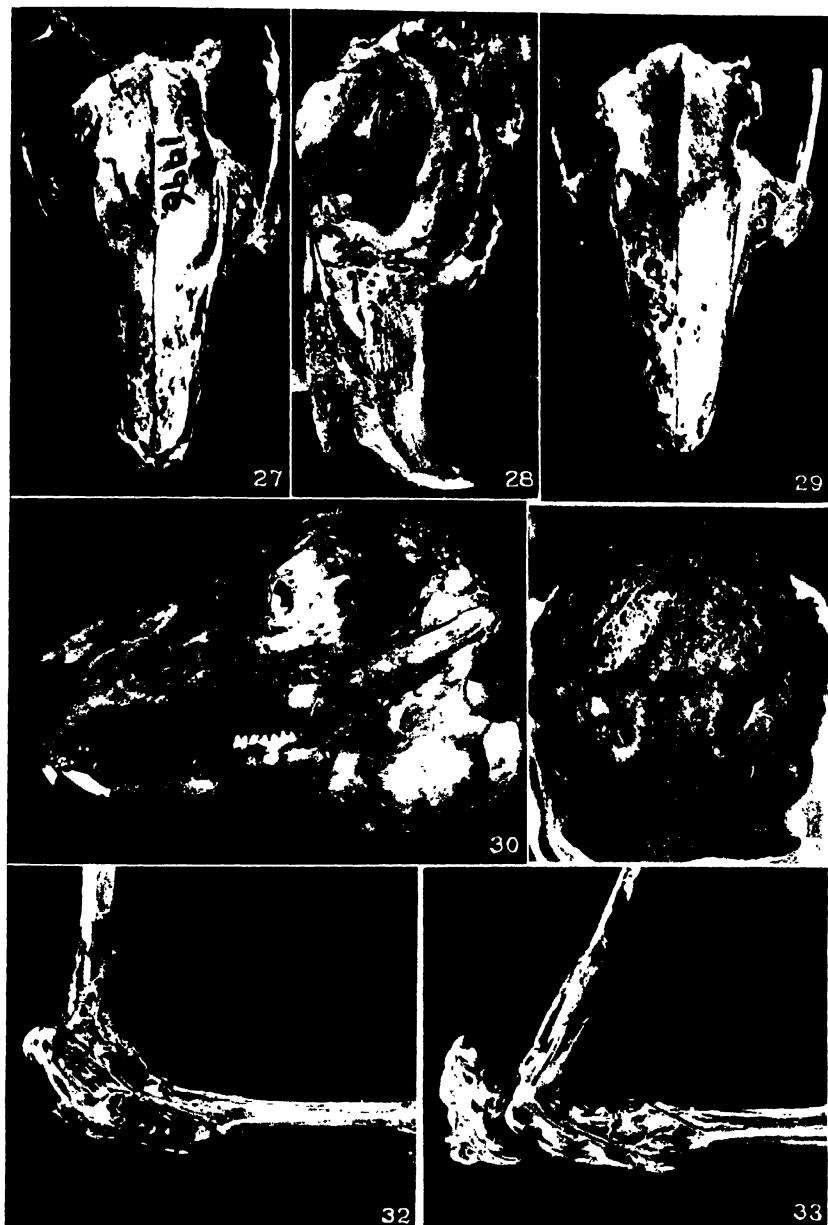
(Brown, Pearce, and Witherbee: Experimental syphilis in the rabbit. VI.)

EXPERIMENTAL SYPHILIS IN THE RABBIT.

VI. AFFECTIONS OF BONE, CARTILAGE, TENDONS, AND SYNOVIAL MEMBRANES.

PART 2. CLINICAL ASPECTS OF SYPHILIS OF THE SKELETAL SYSTEM. AFFECTIONS OF THE FACIAL AND CRANIAL BONES AND THE BONES OF THE FOREARM.

BY WADE H. BROWN, M.D., AND LOUISE PEARCE, M.D., AND WILLIAM D. WITHERBEE, M.D., ROENTGENOLOGIST.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 61 TO 66.

Syphilitic affections of the skeletal system were described in the preceding paper (1) from the standpoint of the pathological process, and it is the purpose of this paper to correlate these changes with clinical manifestations of the disease. This phase of experimental syphilis is of unusual importance on account of the widespread and frequently obscure nature of the infection. The subject will be dealt with, therefore, in somewhat greater detail than other groups of affections with the belief that the facts presented have an important bearing upon latent or obscure infections in the human subject and especially upon congenital forms of the disease.

The conditions to be described are numerous and it will be necessary to divide the material into two papers, the first of which will deal with affections of the facial and cranial bones and the bones of the forearm. The remaining subjects will be considered in the second paper.

Parts Involved and Character of the Affection.

The most obvious affections of the skeletal system were those of the face and of the feet and legs, but lesions were also found in many other parts of the body, such as the cranial bones, the cervical and

caudal vertebræ, the ribs, and the sternum.   The proportion of focal infections which gave rise to clinical signs of disease cannot be estimated, but it seems probable that many obscure infections occurred which did not give rise to any obvious abnormality.

Bone infection was recognized in general by the development of visible or palpable enlargements over the surface of the bones, or, in the absence of these, by rarefication and the loss of structural detail as indicated by the radiograph, and eventually by necrosis and disintegration of the bone.   The skin over the lesion was freely movable, while the nodular mass was firmly attached to the underlying bone. In rare instances, the skin might become adherent, but it is worthy of note that necrosis and ulceration of the skin were never observed in connection with lesions of either tendons or bones, with the exception of the phalanges.   The diagnosis of these conditions as syphilitic affections was readily made by the demonstration of spirochetes in the lesions.

These were the general characteristics presented by all classes of bone infections, but the details of the processes as they occurred in different parts of the body were subject to considerable variation.

Facial and Cranial Bones and Cartilages.

Judged upon the basis of the occurrence of lesions which were sufficiently pronounced to be detected by ordinary methods of examination, the facial bones were more often the seat of syphilitic affections than those of any other part of the body, with the possible exception of the hind feet and legs.   The bones usually involved were the nasals, the two lateral splints composed of the frontal process of the premaxilla and the maxillary process of the frontal, the turbinates, and to a lesser extent the maxillæ, the premaxilla, and the frontal eminences.   These bones lie immediately around the nasomaxillary fossa, and, with few exceptions, the lesions of the facial area were confined to the region of this fossa and the bridge and sides of the nose. The most frequent locations were the bridge of the nose, the end of the nose, and the bony ridge extending from the naso-orbital angle along the sides of the nose, while young animals in particular showed a marked tendency to localization at the point of union of the frontal and maxillary processes.

The appearance presented by animals with well marked facial lesions of various types is shown in Figs. 1 to 7, 11, and 15. Fig. 1 represents a very common type of deformity due to a sharply circumscribed periosteal lesion on the bridge of the nose. Fig. 2 is a more pronounced condition of a similar character which involved the lower two-thirds of the nasal bones and the lateral splints and extended well down on the premaxilla.[1] The profile of the animal shows a gradual curve extending from just below the level of the eyes to the tip of the nose, the deformity being less abrupt than that in the preceding case, and hence not so noticeable at first.

A less common affection is that illustrated in Fig. 3. This was a multinodular lesion of the lower portion of the nasal bones and cartilages and was the most pronounced case of its kind which occurred in our series of animals, measuring 1.85 by 1.7 by 1.2 cm.

Another unusual type which was associated with a marked alteration of the profile is that shown in Fig. 4. The prominence seen above the eye of this animal was due to a large periosteal granuloma involving the supraorbital portion of the frontal bone. This process was bilateral.

The visible deformity in all these animals was quite apparent, but there were many instances in which little or no visible alteration in contour could be detected either on account of the size and form of the lesion or its location. This was especially true when the sides of the nose and the region of the nasomaxillary fossa were involved. Photographic reproduction of even large lesions in these locations was found to be extremely difficult. This class of affections is illustrated by two animals (Figs. 5 and 6) both of which represent very common types of facial involvement.

The lesions of the animal in Fig. 5 consisted of a multinodular mass which extended downward along the bony ridge at the sides of the nose from the region of the naso-orbital angle and then across the bridge of the nose. They were quite large and yet very little of the lateral deformity could be seen until the hair was clipped, disclosing the marked increase in breadth or fullness of the midnasal region which is fairly well shown in the photograph.

In Fig. 6, there is a large, flat, and slightly oval lesion covering the upper half of the right nasal bone and extending well back over the nasomaxillary fossa. It measured more than 0.5 cm. at its center (determined after resection), and yet no deformity of the face was apparent until the hair was removed.

In like manner, the symmetrical deformity produced by many bilateral lesions was even less evident than that of unilateral affections,

[1] See Brown, Pearce, and Witherbee (1), Fig. 28.

especially in the naso-orbital angles or along the upper portions of the nasal splints, which are frequently involved. The appearance presented in the majority of these cases was merely that of a fullness between the eyes or of a slight prominence in the naso-orbital curve.

Facial abnormalities were more apparent with periosteal lesions arising from the outer surface of the bones than with other forms of bone involvement, unless the lesions extended to the outer surface, in which case they frequently assumed an appearance similar to the conditions described.

The extent of the process and the damage produced by infections of the facial bones bore no particular relation to the size of the external growth. These changes could be gauged by palpation, by sounding the lesion with a pointed instrument, or by radiographs. The information to be derived from the use of the latter method of investigation may be illustrated by a series of animals in which the appearance of the lesion, the radiograph, and the bone specimen are compared (Figs. 7 to 16).

The first animal in this series had a marked diffuse periostitis of the nasal bones which produced a typical Roman nose deformity (Fig. 7). By palpation, it was found that the entire lower portion of the bone gave beneath the finger, and a radiograph taken at this time (Fig. 8) showed complete destruction of the mid-area of the nasal bones together with some clouding and loss of architecture in adjacent parts of the bone. After about 2 weeks, the lesion began to subside and regeneration of the bone could be detected. At the end of 6 weeks, a second radiograph (Fig. 9) showed a marked but irregular thickening of the nasal bones and an absence of the usual architecture. There was also a bony mass extending below the level of the nasal bones. The actual condition existing is shown by Fig. 10 which is a photograph of the dried skull.

A second, more significant case is illustrated in Figs. 11 to 14. This was an instance of diffuse periostitis which produced very little facial deformity. A slight thickening could be made out over the bridge and lower portion of the nose and a defect could be detected along the sides of the nasal bones near the end of the nose. The radiograph of these lesions showed very plainly that there was some bone destruction as indicated by the ragged appearance and the thinning out of the under side of the shadow near the end of the nose (cf. Fig. 13 which shows the normal appearance). The fine lines of shadow produced by the turbinates were also blurred, and there were clouding and loss of architecture throughout most of the nasal bone. When these findings were compared with the autopsy specimen (Fig. 14), it was found that the destruction was somewhat greater than might have been imagined from the radiograph.

The late effects following repair in a similar case of diffuse periostitis are shown in Figs. 15 and 16.   In this animal, there was a permanent enlargement of the end of the nose (Fig. 15) due in part to a fibrous thickening of the periosteum and in part to a thickening of the nasal bones as shown in Fig. 16.   The condition had existed for upwards of 2 years.

Infections arising from the interior of the nose were characterized clinically by one or more of three conditions: the presence of a nasal discharge containing spirochetes, necrosis of the outer covering of bone, and finally, the development of external granulomatous lesions. Few of these infections have been recognized clinically and our knowledge of them is mostly that of autopsy findings.   Involvement of both the nasals and the turbinates has been demonstrated in this way, but it will be seen at once that in the absence of any external lesion, there is no simple method of recognizing affections of this class or of differentiating them from affections of the mucous membranes.   It is not improbable, therefore, that many internal lesions may escape detection and that many instances of nasal discharges containing spirochetes and of obstructive phenomena of the nasal passages are referable to bone infection rather than to primary infection of the mucous membranes.   This supposition is supported by abundant pathological evidence.

The probability of the occurrence of lesions other than those described must not be overlooked.   Several instances of peculiar thickening of the frontal and parietal bones of rabbits with generalized syphilis have been noted, and there was one animal with a marked mandibular affection.   These lesions were all inactive, and there was nothing present which would enable one to determine their cause with certainty.   However, a case of necrosis of an occipital condyle was observed, in which the infection was still active. The circumstances in this case will be given later (2).[2]

The points of especial interest in connection with syphilis of the facial bones are the frequency, the location, and the destructiveness of the lesions.   It is also important to note the close analogy which exists between certain of the nasal and supraorbital affections of the experimental animal and those of man, especially in the congenital form of the disease.

[2] Brown, Pearce, and Witherbee (2), p. 531.

Forearms.

.

Bone affections of the anterior extremities of the rabbit were, with one exception, represented by a periostitis involving the distal ends of the ulna and radius. The majority were situated on the extensor surfaces at or near the epiphyseal line, with an occasional lesion at a slightly higher level.

This type was of comparatively common occurrence. As a rule, the lesions were rather small and with the fur intact produced little or no alteration in the appearance of the affected part but were easily detected by palpation. Normally, the extensor surface of the forearm is either perfectly smooth or is marked by small cross ridges and angular projections at the epiphyseal lines and the heads of the radius and ulna respectively. When lesions develop at these points, oval or rounded nodules are formed which are very readily felt on examination and can be seen after removal of the hair.   .

A typical case of bilateral involvement of both ulna and radius is shown in Fig. 17 (*cf.* Fig. 18 in which the left forearm is normal). Three of the four nodes present were distinctly rounded, while that on the right radius was more oval and covered a greater area of the bone.

A second type of affection which was frequently encountered is that seen in Fig. 18. In this instance, there was only one lesion, which formed a rather large oval mass on the right ulna just above the carpus.

In exceptional instances, the lesions of these bones were much more marked than those described and produced very striking deformities, as in Figs. 19 and 20. This, again, was a bilateral affection of both the ulna and the radius. As may be seen from Fig. 20, the lesion of the ulna was very sharply demarcated and raised abruptly from the surface of the bone, while that of the radius was more diffuse and resulted in extensive necrosis of the distal end of the bone.

A second case of an even more marked character is illustrated in Figs. 21 and 22. This was a large oval lesion which arose from the ulna but extended to the radius by way of the interosseous membrane. It was one of the few instances in which definite necrosis of the bones of the forearm could be made out clinically. As shown in the accompanying radiograph (Fig. 22), there was necrosis of the lower end of the ulna as well as the contiguous margins of both the ulna and the radius. The lesion was of very rapid growth, reaching the stage shown within 9 days after it first appeared. The tissues surrounding it were of a violet-red color, and there was a pronounced edema of both the skin and subcutaneous tissues.

In addition to the affections described, mention may be made of an enlargement of the carpus, which was followed by a permanent deformity occurring among the first animals of our series, but the methods then in use were not sufficiently developed to enable us to determine the nature of this condition. From the circumstances, it seems probable, however, that this might have been due to syphilitic infection. No other case of the kind has come under our observation.

The examples of periostitis of the ulna and radius which have been cited were cases in which there was not more than one lesion on each bone. Multiple lesions of one or both bones were occasionally seen, but they were usually small. In many instances, only one forearm was affected, but more often the involvement was bilateral and symmetrical.

It was a notable feature of this group of lesions that they rarely led to extensive necrosis. Grossly, the surface of the bone was eroded or roughened, and obliteration or widening of the epiphyseal line could be demonstrated by use of x-rays. Similarly, when the lesion healed, the bone might show a slight roughening or increase in thickness with a few tiny nodes (see lateral margin of the ulna in Fig. 22), but rarely was there any considerable deviation from the normal. Microscopic examination showed, however, that the syphilitic process frequently extended through the entire thickness of the bone and that the effect produced was in general much greater than gross appearances would indicate.

Other aspects of the subject of syphilis of the skeletal system will be presented in Part 3 of this paper (2).

BIBLIOGRAPHY.

1. Brown, W. H., Pearce, L., and Witherbee, W. D., *J. Exp. Med.*, 1921, xxxiii, 495.
2. Brown, W. H., Pearce, L., and Witherbee, W. D., *J. Exp. Med.*, 1921, xxxiii, 525.

EXPLANATION OF PLATES.

All illustrations are reproductions of photographs or radiographs which have not been retouched and represent objects at their natural size. Statements of time are estimated from the date of inoculation unless otherwise indicated.

Figs. 1 to 6. Appearance presented by animals with syphilitic affections of the facial and cranial bones.

PLATE 61.

Fig. 1. 146 days.   Nodular periostitis, bridge of the nose.

Fig. 2. 105 days.   Marked diffuse periostitis of the lower two-thirds of the nasal bones.[1]

Fig. 3. 159 days.   Marked nodular periostitis and perichondritis of the nasal bones and cartilages.

Fig. 4. 71 days.   Marked periostitis of the supraorbital region.

PLATE 62.

Fig. 5. 110 days.   Multinodular periostitis of the nasomaxillary fossa, the sides and bridge of the nose.   The lesions were very pronounced.

Fig. 6. 92 days.   Periostitis of the right nasal bone and splint.   The lesion formed a broad flattened mass which covered the upper half of the bone and adjacent portions of the fossa.

Figs. 7 to 16. A comparative study of clinical and pathological conditions in syphilitic affections of the nasal region.

PLATE 63.

Figs. 7 to 10. Marked diffuse periostitis of the nasal bones and splints with necrosis and regeneration.

Fig. 7. 68 days.   The deformity of the nose.   The lesion was centered over the bridge of the nose and extended from just below the level of the eyes to the tip of the nose.

Fig. 8. 68 days.   Radiograph showing complete necrosis of the bone at the center of the process.   The turbinates are also involved.

Fig. 9. 6 weeks later.   Regeneration of bone.   The nasal bones are considerably thickened, irregular, and devoid of architecture.   There is also an irregular osteoid mass beneath the nasal plates.

Fig. 10. 121 days.   Lateral view of the skull showing the regenerated bones.[3]

---

[3] For frontal view see Brown, Pearce, and Witherbee (1), Fig. 29.

PLATE 64.

FIGS. 11, 12, and 14. Diffuse periostitis which produced very little alteration in the facial contour.

FIG. 11. 102 days. A slight prominence towards the end of the nose was the only visible abnormality. Palpation showed necrosis of the margins of the nasal bones.

FIG. 12. 116 days. Radiograph showing clouding of the nasal bones and erosion of the under side near the end of the nose. There are also a marked clouding and loss of finer shadow details throughout the nasal chambers and parts of the maxilla and premaxilla, indicating an extensive involvement of these parts.

FIG. 13. Normal radiograph for comparison.

FIG. 14. 123 days. Skull of the animal in Figs. 11 and 12.[4]

PLATE 65.

FIGS. 15 and 16. 2 years and 2½ months. Chronic fibrous periostitis with bulbous thickening of the end of the nose—a permanent condition. The enlargement of the nose was due in part to fibrous tissue and in part to thickening of the bone. In the radiograph (Fig. 16) note the change in shape and prolongation of the nasal bones, eburnation, and increase in osteoid tissue on the under side of the nasal bones and throughout the nasal region.[5]

FIGS. 17 to 22. Syphilitic affections of the radius and ulna.

FIG. 17. 61 days. A typical bilateral periostitis of the radius and ulna.

FIG. 18. 92 days. A typical case of unilateral periostitis of the ulna (right) with extension to the interosseous membrane and the adjacent margin of the radius. The left forearm is normal.

PLATE 66.

FIG. 19. 76 days. An unusually marked affection of both the ulna and radius. The condition was bilateral.

FIG. 20. 77 days. Autopsy specimen of the same lesions. That of the ulna is seen to be a sharply demarcated periosteal granuloma, while that of the radius is an osteitis of the head of the bone.

FIG. 21. 60 days. Marked periostitis and osteitis of the distal ends of the ulna and radius.

FIG. 22. 61 days. Radiograph of the same lesions. Note the irregularities and rarefication of the lower ends of the bone and the fusiform enlargement of the radius.

---

[4] For frontal view see Brown, Pearce, and Witherbee (1), Fig. 27.

[5] For skull see Brown, Pearce, and Witherbee (1), Figs. 6 and 7.

PLATE 61.
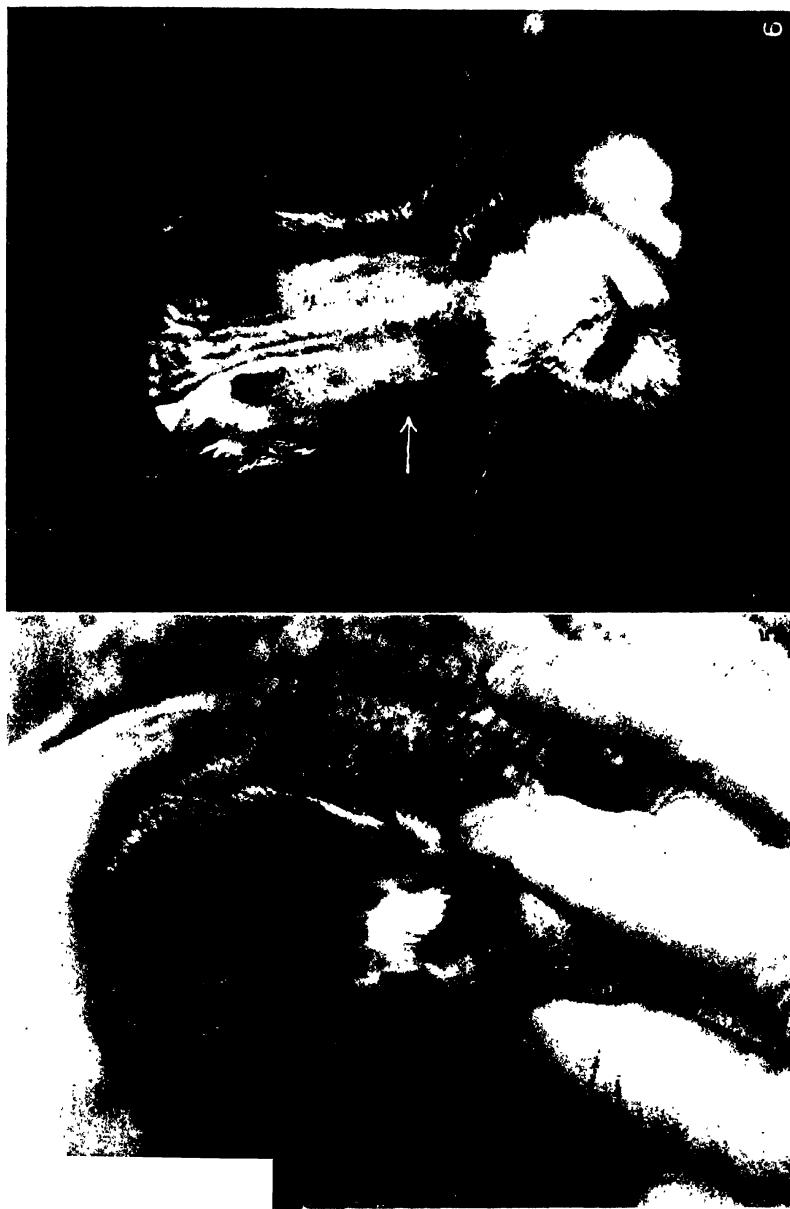
(Brown, Pearce, and Witherbee: Experimental syphilis in the rabbit. VI.)

8

9

(Brown, Pearce, and Witherbee: Experimental syphilis in the rabbit. VI.)

11
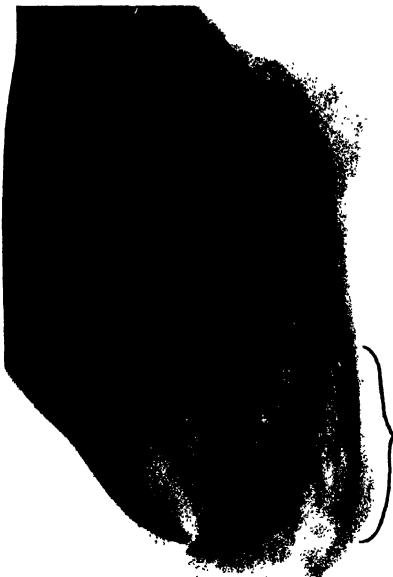
12

13

14
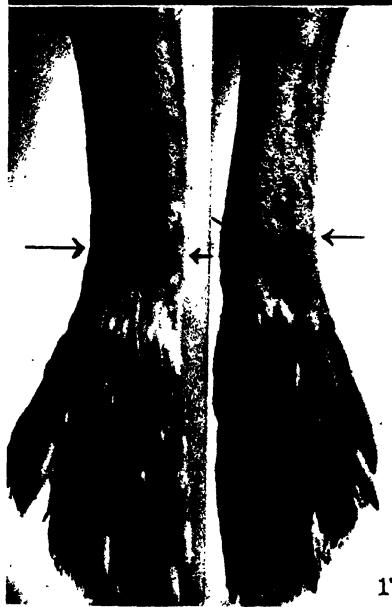
21   22

EXPERIMENTAL SYPHILIS IN THE RABBIT.

VI. AFFECTIONS OF BONE, CARTILAGE, TENDONS, AND SYNOVIAL MEMBRANES.

PART 3. SYPHILIS OF THE POSTERIOR EXTREMITIES WITH OTHER AFFECTIONS OF A MISCELLANEOUS TYPE.

BY WADE H. BROWN, M.D., AND LOUISE PEARCE, M.D., AND WILLIAM D WITHERBEE, M.D., ROENTGENOLOGIST.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 67 TO 72.

The phases of syphilis of the skeletal and supporting systems to be considered in this paper are the affections of the hind feet and legs, and of the vertebral column, joint cavities, and tendons with the clinical history of this form of experimental syphilis.

Hind Feet and Legs.

In our first series of animals, there were fewer instances of involvement of the bones of the hind feet and legs than of the forearm, which was the reverse of the condition noted with the cutaneous lesions. Among the more recent cases of generalized syphilis, however, the incidence of bone lesions of the posterior extremities was markedly increased, and the lesions themselves were much more pronounced. The bones involved were the lower end of the tibiofibular, the tarsals, the metatarsals, and the phalanges. The affections included both periosteal and endosteal processes with a considerable number of cases resulting in epiphyseal separation or pathological fracture.

Tibiofibular.—The most easily recognizable lesions of the lower extremities were those of the tibiofibular, all of which were periosteal in origin and with a single exception occupied essentially the same position; namely, the anterolateral surface of the lower end of the bone, or what might be termed the external malleolus. This position

57

corresponds to the line of union between the tibia and the fibula and normally is marked by a small depression surrounded by a series of angular points or ridges. The development of a periosteal lesion in this location caused a filling in of the depression and rounding off of the bony prominences.

Affections of this type were quite common. Some of the lesions were of a comparatively small size, while others reached a centimeter or more in diameter and produced a very characteristic deformity, as may be seen by reference to Figs. 1 to 3.

In this animal, there was a bilateral involvement of the bones, the lesions of the two sides being of approximately the same size and character. The right leg (Fig. 1) illustrates the appearance presented with the fur intact, while the left, with the hair removed, shows more accurately the location of the lesion and the actual deformity produced. The autopsy preparation in Fig. 2 shows the appearance with the skin reflected.

None of the affections of this group appeared to produce any considerable gross alteration in the bone. The changes noted were analogous to those of the ulna and radius. Fig. 3, which is a radiograph of the lesions in Fig. 1, shows the condition usually found. On the right, there is a very definite thinning of the outer table and some irregularity in the surface of the bone. The surface of the left tibia is less affected and shows no more than a suggestion of a roughening, but there appears to be some rarefication in the lower end of the bone, and the most marked alterations were probably in the heads of the bones. This radiograph may also be used as a means of orientation for the lesions and the parts of the bone affected.

Minor variations in the location of the lesions were not uncommon, but in only one instance was the position materially different from that described. In addition to the usual involvement of the external malleolus, this animal developed a third lesion which took the form of an annular periostitis surrounding the shaft of the right tibiofibular over an area about 1 cm. in breadth and at a level of 2 to 3 cm. above the distal end of the bone.

Metatarsals.—While affection of the metatarsals was of frequent occurrence, the lesions were, with few exceptions, confined to the so called fifth or lateral metatarsal; the other bones involved were the first and the fourth. This group of affections presented a variety of conditions consisting chiefly of periosteal lesions but including also affections of the bone and a considerable number of cases of epiphyseal

separation. While some were small and rather difficult to detect, except by the most careful palpation, the usual affection was quite obvious to one familiar with the topography of this part of the foot.

The fifth metatarsal of the rabbit is much the same as that of man. The features of especial importance are the presence of a sharp bony prominence, or hook, formed by the tuberosity at the proximal end of the bone and the narrow tendinous ridge which extends along the lateral margin of the foot throughout the length of the bone. These features of the normal foot are shown in Fig. 4.

The tuberosity, or proximal end, of the metatarsal was perhaps most frequently affected. Irrespective of its origin, the condition usually produced was of the nature of a firm swelling which centered about the head of the bone and extended some distance along the adjacent portion of the shaft. The picture presented by such affections was that shown in Fig. 5. The bone injury produced in this case is shown in Fig. 6 which is a radiograph of the feet in Figs. 4 and 5.

Occasionally, much greater proportions and a more destructive character were noted; an example of this kind is given in Figs. 7 and 8. As in the preceding case, the lesion arose from the head of the fifth metatarsal but extended to the adjacent portions of the fourth metatarsal (Fig. 8); there was practically complete necrosis of the head of the fifth metatarsal followed by the formation of an irregular bony mass which is well shown in the radiograph (Fig. 8).[1]

A second group of lesions occurred along the shaft of the bone either singly or as multiple nodules. The majority of these were periosteal affections; they were usually located on the lateral or dorsal surface but were occasionally found on the plantar surface or completely encircled the bone. The usual form was that of a fusiform swelling such as is shown in Fig. 9. A more pronounced case of a similar character is seen in Fig. 10. In this instance, there were two distinct lesions present, one of which appeared as a spherical mass embracing the head of the metatarsal and the other as a fusiform enlargement extending the full length of the shaft. The condition was bilateral and symmetrical.

[1] Fig. 16 is taken from the same animal.

None of the periosteal lesions of the shaft thus far examined have resulted in a gross alteration which amounted to more than a slight erosion of the bone or slight roughening and thickening which persisted for some time after the healing of the lesion (see Fig. 21). Microscopic changes, however, were more marked.

In a few instances, affections of the bone or medullary cavity were discovered by x-ray examinations and verified by autopsy, but very little is known of their clinical history. At times, they produced conditions analogous to those of periostitis or resulted in necrosis and pathological fracture.

A third group of lesions arose from the distal end of the metatarsal, and while many of these were simple periosteal processes analogous to those of the shaft and the proximal end of the bone, they were of peculiar interest on account of the frequent occurrence of pathological fractures or epiphyseal separations. At present, we cannot attempt to differentiate between the various types of pathological processes which were concerned in the production of these conditions. Some were obviously the result of periosteal infections, while others appeared to be due to infection within the bone itself or to a combination of the two processes. An example of each of these may be given.

The lesion which is illustrated in Figs. 11 to 13 began as a simple periosteal process, and the first radiograph showed little if any alteration in the bone. Growth was unusually active, however, and within a week, slight crepitus could be detected and the radiograph showed the presence of a fracture of the neck just proximal to the epiphyseal line (Fig. 13). It could not be determined whether this was due entirely to the lesion of the periosteum or to the presence of a second focus of infection within the bone which was obscured by the periosteal lesion. When the animal was killed 11 days later, the bone was found to be irregularly and markedly thickened in its outer third.

The second case, illustrated in Figs. 14 and 15, is what appears to be a clear instance of an epiphyseal infection, or an osteochondritis, and is of especial interest on account of its occurrence in an adult animal. When first noted, there was a firm narrow ridge at the epiphyseal line which was barely palpable and could not be diagnosed with certainty as a syphilitic lesion. This ridge increased slightly, and within a few days, an edematous swelling appeared, the extent of which is indicated by the shadows in the radiograph taken at this time (Fig. 14). There was a distinct crepitus, and the radiograph showed an obliteration of the epiphyseal line with a clean-cut separation of the epiphysis (Fig. 14). The lesions healed with a slight bony thickening, and it will be noted that the epiphyseal line was not reestablished (Fig. 15).

From the description of metatarsal lesions which has been given, it will be seen that the essential differences between affections of these bones and those of the tibiofibular or ulna and radius were the extent of the bone involvement and the occurrence of focal infections within the bone and especially at lines of epiphyseal union.

Tarsus.—The status of syphilitic affections involving the tarsus is still somewhat obscure. As was pointed out in Part 1 of this paper (1), there is a variety of conditions affecting this part of the body which present much the same clinical picture. Those observed were chiefly affections of the calcaneus and included a few cases of syphilitic periostitis with numerous instances of necrosis of the calcaneus, pathological fracture, and epiphyseal separation together with some instances of extension of the infection to other bones of the tarsus or to ligaments, tendons, and synovial membranes.

All the periosteal lesions of the calcaneus were situated on the dorsal or superior surface of the bone near the anterior end. Normally, there is a well marked depression at this point which, in cases of periostitis, was obliterated or filled by a firm elastic mass. The resulting alteration in appearance is shown in Figs. 11 and 12, which may be compared with other illustrations.

So far as we are aware, in no instance was any marked necrosis of the underlying bone produced, but it is important to note that the majority of the cases of necrosis of the calcaneus which occurred in the absence of any known lesion of the periosteum occupied exactly the same position. This is clearly illustrated in Fig. 16.

The conditions designated as necrosis of the calcaneus, pathological fracture, and separation of the epiphysis have been considered in detail elsewhere (1). It seems necessary only to recall that all presented a common symptom-complex, the outstanding features of which were the sudden development of lameness, an edematous swelling of the tarsus, tenderness, and crepitus, the cause for which could be determined with accuracy only by radiograph or by careful dissection. The swelling and edema are well shown in Fig. 16.

In a few instances, other bones than the calcaneus have been found to be involved in affections of the type described and as a result of extension of lesions from such points as the head of the metatarsals (Fig. 16). However, they presented no distinctive clinical characteristics.

Phalanges.—As one passes from the larger and more accessible bony structures to those which are extremely small or difficult of examina- · tion, the question of the presence or absence of bone lesions and the nature of the injury become less certain. This applies to syphilitic affections of the phalanges and the bones of the carpus and tarsus as well. However, numerous instances of periostitis of the phalanges, or dactylitis, and of necrosis of the bones with no demonstrable lesion of the periosteum have been observed in the toes of the hind feet.

The occurrence of affections of the terminal phalanges which gave rise to abnormalities of the nails was referred to in connection with the cutaneous manifestations of syphilis (2, 3).

Lesions of this type are shown in Figs. 17 to 19 and also on the fifth toe in Fig. 8. There is a periostitis of the terminal phalanges of several of the toes in Figs. 17 and 18, and the loss of the nail can be traced in the fourth toe of the left foot. Fig. 19 shows a more extreme condition, which was permanent. Enlargement and induration of the terminal phalanx, as in these cases, rarely occurred without the production of an easily recognizable paronychia.

Recorded instances of a simple periostitis of the phalanges which did not involve the skin or its appendages were comparatively few. However, a large proportion of these cases occurred on the fifth or lateral toe as in Figs. 20 and 21, thus conforming to the order of distribution recorded for other lesions of the hind feet. In this connection, attention may be called to a striking tendency towards the occurrence of serial periosteal lesions along the outer sides of the feet, producing a beaded effect which is well shown in Fig. 21.

Lesions were observed on the first, second, and third phalanx; some produced no appreciable alteration in the bone, while others resulted in necrosis which was occasionally followed by fracture or disintegration.

Infections arising within the bone were discovered in a few animals, and their development was followed by serial radiographs. There were no appreciable external evidences of bone involvement in these cases until necrosis and disintegration or fracture of the bone occurred. The parts then became more or less swollen and tender, and in some instances a crepitus could be detected.

Several examples of affections of this kind may be seen in Figs. 16 to 18. The lesion in the first phalanx of the outer toe of the right foot in Figs. 17 and 18 is brought out especially well. By careful inspection (use reading glass), it will be seen that there is an enlargement of the distal end of this phalanx (Fig. 17) with rarefication and loss of architecture, but that there is no shadow to indicate the presence of a periosteal lesion. 13 days later (Fig. 18), a fracture occurred at this point accompanied by the usual reaction in the surrounding tissues.

There were a number of definite bone lesions present in the feet of this animal, none of which could have been clearly recognized except by radiographic examination; there were periosteal lesions of several toes (outwardly paronychias), and osteitis in the phalanx mentioned and at both extremities of the fifth metatarsal of the same foot. In Fig. 16, a number of the phalanges are also seen to be involved. This will serve to indicate the difficulty in the recognition of lesions of this type, our knowledge of which is at present rather limited.

Obscure Bone Affections.

The conditions which have been described in the preceding pages may with reason be regarded as the obvious bone affections in contradistinction to infections which are accompanied by no signs which are sufficiently distinctive to enable one to recognize them clinically or even to suggest the possibility of their existence. It is quite certain that such conditions do occur as indicated by the frequency with which affections of the interior of the nares and of the small bones of the feet have been discovered by radiographic or pathological examination.

Other positions in which we have reason to believe that obscure affections may exist are the vertebral column, the cranial bones, the sternum, the ribs, and the mandibles. In each of these sites, lesions have been found whose pathology was sufficient to identify them as syphilitic. Infection of the caudal vertebræ has been recognized clinically in several animals, the condition being manifested as fusiform swellings. In other instances where no injury was suspected, necrosis of the bones was demonstrated by radiograph.

Our attention was attracted to two cases of infection of the cervical vertebræ by the development of symptoms of meningitis. In one of these animals, postmortem examination revealed the presence of necrosis of the cervical vertebræ, and spirochetes were demonstrated in the cerebrospinal fluid. In the other, the upper cervical vertebræ

and one of the occipital condyles were involved. Histologically, these bones showed a typical syphilitic reaction in the Haversian canals and marrow spaces, with absorption and necrosis of bone.

In two instances, lesions were discovered at autopsy at the costo-chondral junction of one of the ribs which corresponded grossly and histologically with other bone lesions in the animals. In another animal, a healed lesion was found in the xiphoid and the adjacent part of the body of the sternum which appeared to be of syphilitic origin, and a similar condition was observed in the mandible of another animal.

The occurrence of obscure bone affections and infections associated with very slight local reaction is of considerable importance and lends support to the belief that many cases of latent or obscure human infection may find their explanation in the occurrence of an analogous group of conditions.

Tendons and Synovial Membranes.

In Part 1 of this paper (1), mention was made of the occurrence of affections of tendons, joints, and synovial membranes. With the exception of the tendons, these conditions were not accompanied by signs which were sufficiently distinctive to permit their clinical recognition, and nothing can be said of their clinical history.

Only a few cases of primary tendon involvement were recognized clinically, all of which occurred in the tendo achillis. The lesions were usually small nodular, or fusiform masses which could be detected only by palpation. In one instance, however, the lesion developed on the outer side of the tendon and produced a visible enlargement over which the skin was freely movable.

Clinical History of Bone Affections.

The clinical history of bone affections cannot be given in so complete form as one would wish, since only a few animals of this group were held for any considerable period of time; our knowledge is, therefore, chiefly of the early stages of the infection.

From available data, it would appear that syphilis of the bones occupies a position with relation to the infection as a whole which is closely analogous to that of the cutaneous manifestations.

Among conditions which seemed to exercise a predisposing influence upon the occurrence of bone infection, mention may be made of the reduction or early suppression of primary lesions by unilateral instead of bilateral inoculation, by castration or excision of lesions, or by the use of therapeutic agents. When procedures of this kind were employed, the relative incidence as well as the extent of the bone involvement was definitely increased. The peculiarities of distribution and the marked predilection for unprotected bony prominences would indicate that the factor of trauma also plays a part both in the occurrence and in the localization of this class of lesions.

The important point to be noted, however, is that lesions of periosteum and bone appear to occupy a definite position in the scheme of defensive reactions in the experimental animal. Initially, this reaction is bound up with the development of the primary lesions, but if the latter are suppressed at an early stage of the infection, it is almost invariably taken up by the periosteum and bone. Conversely, if the primary lesions are allowed to progress uninterruptedly, or if they are suppressed late in the course of the infection, protection is apt to be conferred upon this group of tissues, and the burden of reaction is assumed by some other set of tissues such as the skin or the mucous membranes. The detailed consideration of the facts upon which this statement is based will be taken up elsewhere (4).

Time of Occurrence.—From what has been said, it may be surmised that the time of appearance of bone lesions varied greatly. Under conditions which favored their development, they were among the earliest manifestations of a generalized disease. While the extreme limits of variation in our series of animals were from a minimum of 38 days to $15\frac{1}{2}$ months after inoculation, most often the lesions appeared within 2 to 3 months. The reactive period of bone infection, if we may term it such, was shorter and more sharply demarcated than that of other lesions. This was especially true in animals whose primary lesions had been suppressed. In these, the lesions appeared with great regularity at or near the expiration of the 60 day period following inoculation, those of the nasal region as a rule being recognized slightly earlier than those of other bones.

Course and Duration.—The course of the bone lesions was one of comparatively rapid development and rapid decline, in which there

was less of the cyclic variation to be seen than in almost any other class of syphilitic affections. Clearly defined periods of growth and regression were observed in some animals, but it is worthy of note that no instance of true relapse or recurrence of a healed lesion was recorded. In a single instance, in which a periosteal lesion on the nose of an animal persisted for more than a year, there were several periods of marked regression followed by renewed activity; this was the nearest approach to true relapse which was observed. Whether these peculiarities of bone lesions may be attributed to difficulties of observation on the one hand and, in our series, to the short period of observation on the other, or are characteristic of this group of affections cannot be said.

As a rule, the period of active infection was comparatively short. Some lesions disappeared within a few weeks, and few lasted for more than 1 to 2 months. The process of repair in cases of marked bone destruction was obviously more prolonged. In exceptional instances, active lesions persisted for several months; the longest recorded period in our series was 20 months, and this lesion was still active when the animal was killed.

Gross evidences of bone injury were, as a rule, comparatively slight and tended to disappear; in the case of the more destructive lesions, however, repair was less perfect and more or less permanent alterations were produced which were akin to those seen in syphilitic infections of man.

CONCLUSIONS.

The facts presented above indicate clearly that localized infections of periosteum, bone, cartilage, tendons, and synovial membranes form an important group of affections in rabbits inoculated with suitable strains of *Treponema pallidum*.

Among the more important features of this group, mention may be made of the early occurrence of the lesions and their relation to other manifestations of disease, the involvement of bones in exposed locations or of bones which are subject to pressure or trauma, the high incidence and destructiveness of affections of the nasal region, the tendency to localization at points of bony union or at epiphyseal lines, and the frequency of obscure focal lesions.

From a general standpoint, it will be seen that syphilis of the skeletal system of the rabbit is a very characteristic condition and that it combines features of both the acquired and the inherited form of the disease in man. It cannot be said that the two conditions are identical, yet they possess many fundamental characteristics in common, and it is believed that this form of experimental syphilis is of more than usual importance on account of the opportunity which it affords for the study of lesions of the skeletal system.

SUMMARY.

A systematic study was made of the affections of bone, cartilage, tendons, and synovial membranes which occurred in a series of rabbits with generalized syphilis. Localized infection of this group of structures was found to be of very frequent occurrence. The parts involved were, in the order of their frequency, the facial and cranial bones and cartilages, the bones, tendons, and joints of the feet and legs, the cervical and caudal vertebræ, the ribs, and the sternum. These infections often gave rise to characteristic manifestations of disease which could be detected without difficulty by inspection or palpation of the part. In many instances, however, clinical manifestations were so slight that the presence of lesions could be detected only by radiographic or pathological examination.

Detailed descriptions of various clinical types of disease were given and the clinical manifestations correlated with the pathological process. It was pointed out that bone lesions exhibited a decided predilection for certain exposed bony prominences, for lines of bony union, and for epiphyseal lines in particular.

A study of the clinical history of bone lesions brought out the fact that they were among the earliest of the generalized forms of disease; they tended to pursue a comparatively rapid course, and relapse was never observed.

Especial emphasis was laid upon three aspects of the experimental infection: the analogy existing between certain forms of the animal and human affections, the relation of syphilis of the osseous system to other evidences of disease, and the occurrence of obscure bone lesions.

In this connection, it was pointed out that the nasal and epiphyseal lesions of the rabbit presented a striking analogy to those of congenital syphilis in man.

It was also pointed out that syphilis of the osseous system occupied a definite position in the scheme of defensive reactions such that lesions of these tissues might be favored or inhibited according to the experimental conditions employed.

Finally, the frequency with which infections occurred which were not accompanied by sufficiently distinctive signs even to suggest the possibility of their existence was interpreted as evidence that some cases of latent or obscure infection in man might find their explanation in the presence of a similar group of affections.

BIBLIOGRAPHY.

1. Brown, W. H., Pearce, L., and Witherbee, W. D., *J. Exp. Med.*, 1921, xxxiii, 495.
2. Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1920, xxxii, 445.
3. Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1920, xxxii, 473.
4. Brown, W. H., and Pearce, L., *Arch. Dermatol. and Syphilol.*, 1921, iii, 254.

EXPLANATION OF PLATES.

All illustrations are reproductions of photographs or radiographs which have not been retouched and represent objects at their natural size. Statements of time are estimated from the date of inoculation unless otherwise indicated.

PLATE 67.

FIGS. 1 to 3. Syphilitic affections of the tibiofibular, or external malleolus.

FIG. 1. 84 days. A typical case of bilateral, nodular periostitis of the external malleolus.

FIG. 2. Autopsy specimen of the left leg showing the almost spherical granulomatous mass with some extension to the surrounding tissues.

FIG. 3. Radiograph showing location of the lesions in Fig. 1 with reference to bony parts and the apparent effect upon the underlying bone. Note the slight alteration in the margin of the shaft of the left tibia as compared with the right. The most marked changes are in the head of the bones (indicated by arrows). The lighter shadows in the left tibia are possibly due to lesions within the bone.

FIG. 4. Appearance of normal metatarsal.

FIGS. 5 to 15. Syphilitic affections of the metatarsals.

PLATE 68.

FIGS. 5 to 8. Lesions of the proximal end of the metatarsals.

FIG. 4. Lateral view of right hind foot showing normal topography of the metatarsal area.

FIG. 5. 135 days. Left foot of same animal showing a typical enlargement of the head and adjacent portions of the shaft of the fifth metatarsal due to syphilitic involvement of this portion of the bone.

FIG. 6. Radiographs of the feet shown in Figs. 4 and 5. Note the comparative condition of the proximal portions of the lateral metatarsals.

FIG. 7. 83 days. A marked syphilitic affection of the proximal ends of the fourth and fifth metatarsals.

FIG. 8. Radiograph showing the extent of the bone involvement.

PLATE 69.

FIGS. 9 to 13. Affections of the shaft and distal ends of the metatarsals.

FIG. 9. 117 days. A fusiform lesion of the shaft of the lateral metatarsal. Periostitis.

FIG. 10. 93 days. A marked periosteal affection of the entire fifth metatarsal. There is a nodular granulomatous lesion at the proximal end of the bone and a fusiform lesion extending its full length; this lesion completely surrounded the bone. There is also a skin lesion over the tarsus and just above this, a periosteal lesion of the tibiofibular.

FIGS. 11 and 12. 92 days. Dorsal and lateral views of a marked affection of the distal end of the fifth metatarsal. These photographs also show a prominent periosteal granuloma on the dorsal surface of the anterior end of the calcaneus (marked by arrow).

FIG. 13. Radiograph showing pathological fracture of the shaft of the metatarsal resulting from the lesion shown in Figs. 11 and 12.

PLATE 70.

FIGS. 14 and 15. Affections of the distal ends of the metatarsals.

FIG. 16. Affection of the tarsus.

FIG. 14. 58 days. An epiphyseal separation of the distal end of the fifth metatarsals. A displacement of the shafts and epiphyses is seen; a light line or band is seen crossing the bone in the position of the epiphyseal line, and a small spur of bone projects from the outside of both metatarsals. Lesions marked by arrows.

FIG. 15. 6 weeks later. Repair of the epiphyseal lesions. Note the general enlargement of the distal ends of the bones (lateral metatarsals), the loss of epiphyseal lines, and the thickening of the bones at the site of the lesions. Adjacent areas of the fourth metatarsals also appear to be slightly altered.

FIG. 16. 84 days.   An unusually pronounced case of bone syphilis.   There is necrosis of the right calcaneus associated with effusion into the surrounding tissues and swelling of the tarsus, foot, and leg.   Necrosis of the left lateral metatarsal is also present, with effusion and swelling of the foot and tarsus, and a number of lesions may be seen in the phalanges of both feet (marked by arrows).   Pathological fracture of several of these bones occurred a few days later.

FIGS. 17 to 21.   Affections of the metatarsals and phalanges.

PLATE 71.

FIG. 17. 92 days.   Periostitis and osteitis.   Left foot: periostitis of all three phalanges of the fifth toe and of the second and third phalanges of the fourth toe. Right foot: periostitis of the terminal phalanges of the third toe and of the shaft of the lateral metatarsal; osteitis of the proximal and distal ends of the metatarsal (note absence of epiphyseal line) and of the distal end of the first phalanx of the fifth toe.   Other shadows are from cutaneous lesions.

FIG. 18. 2 weeks later.   Left foot: necrosis and buckling of the second phalanx of the fifth toe and loss of nail of the fourth toe.   Right foot: necrosis and fracture of first phalanx of fifth toe.

FIG. 19. 1 year and 9 months.   Marked permanent lesions of the phalanges.

PLATE 72.

FIGS. 20 and 21. Symmetrical affections of the lateral metatarsals and phalanges showing distributional tendencies.

FIG. 20. 85 days.   Periosteal lesions on the lateral surface of the feet extending from the base of the metatarsals to the terminal phalanges.   Other toes are slightly affected, notably the fourth toe of the left foot.

FIG. 21. 85 days.   Serial lesions of the metatarsals and phalanges illustrating the bead-like effect produced in the rabbit's foot and various degrees of bone involvement associated with them.[2]

---

[2] See also Brown, Pearce, and Witherbee (1), Fig. 3.
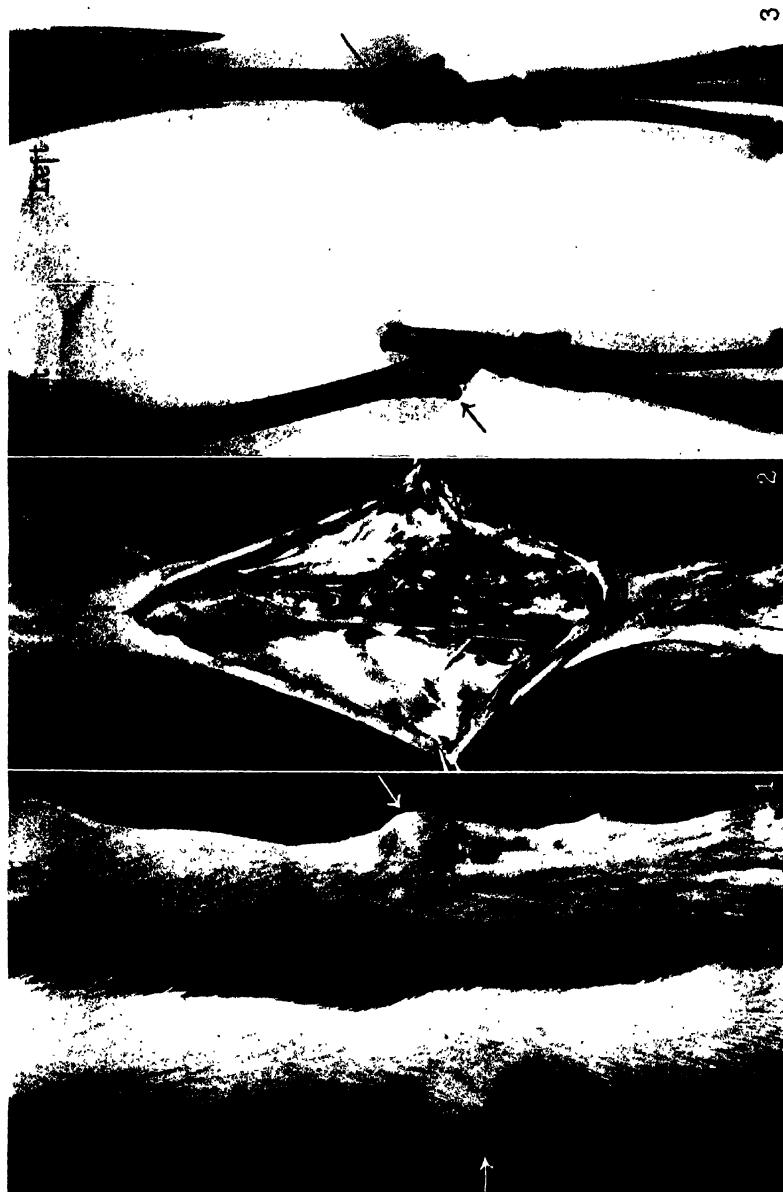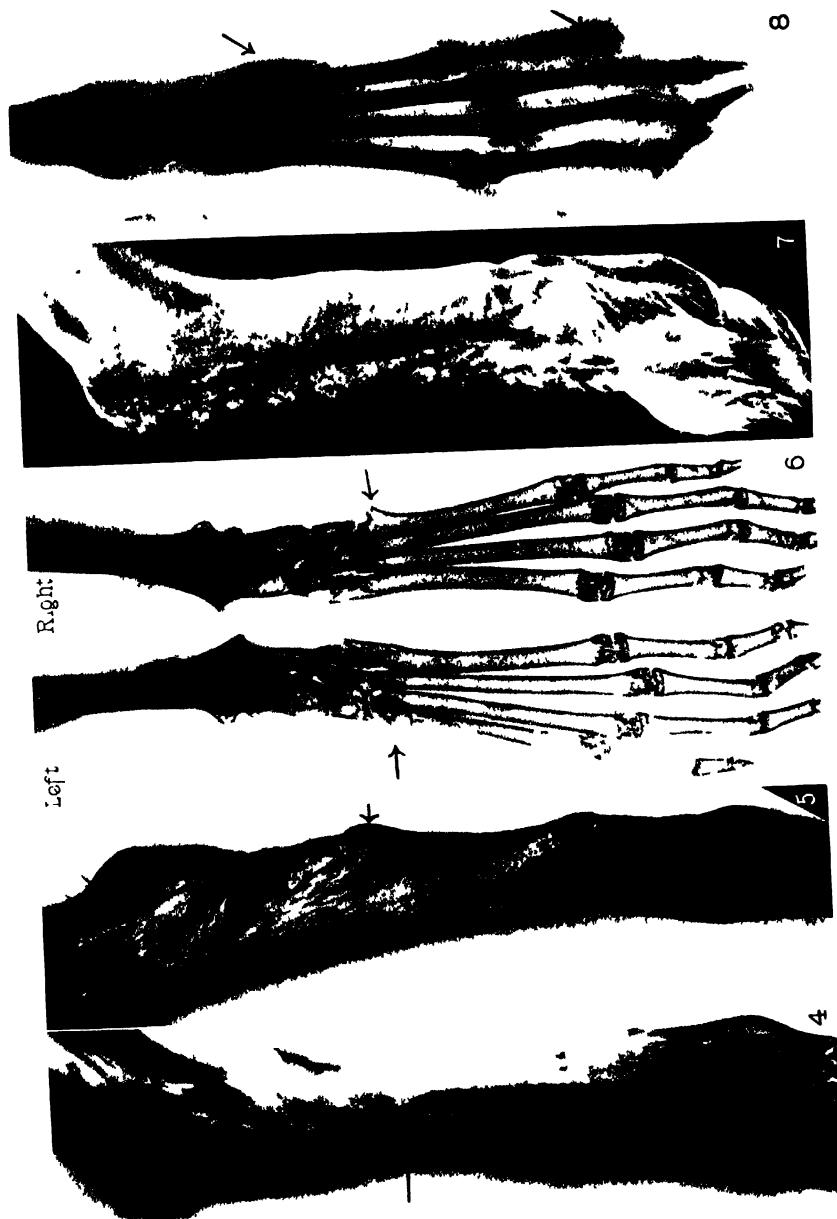
PLATE 68

(Brown, Pearce, and Witherbee: Experimental syphilis in the rabbit.   VI')

PLATE 72.

20
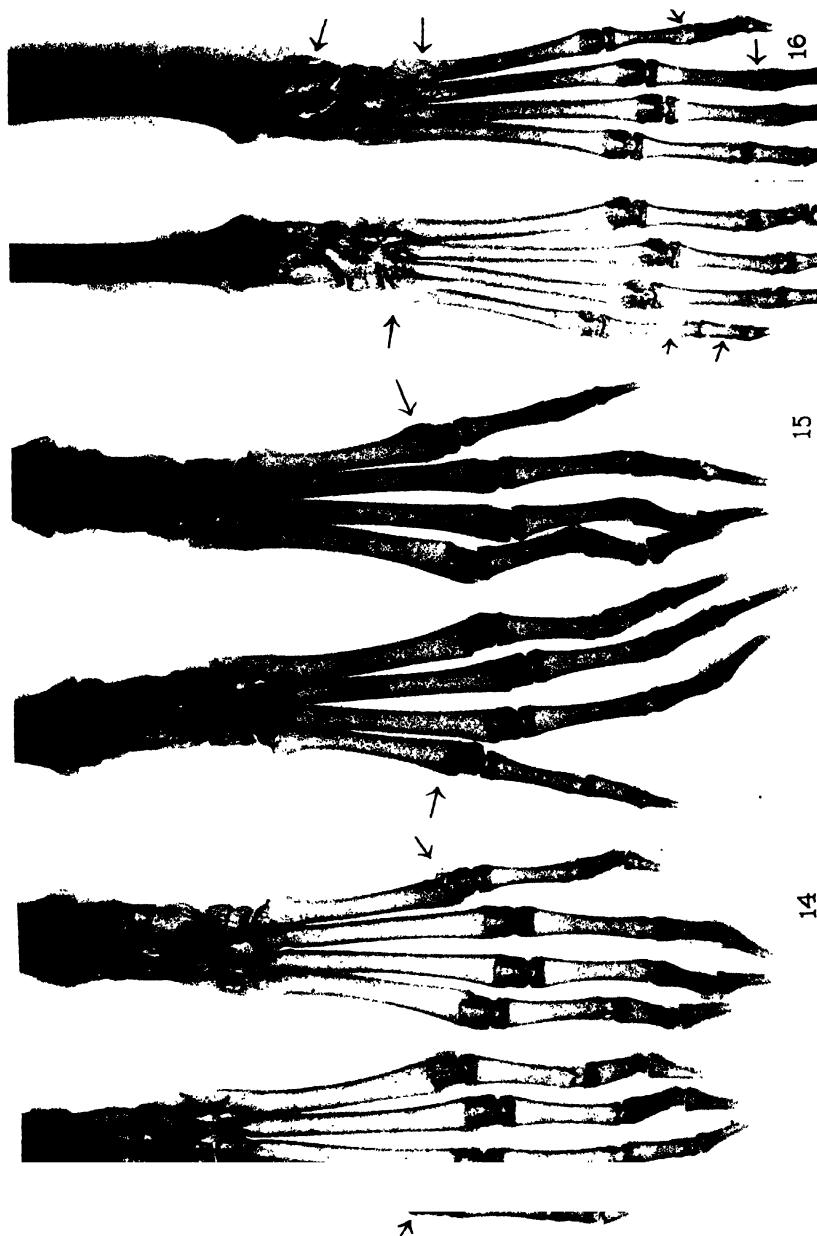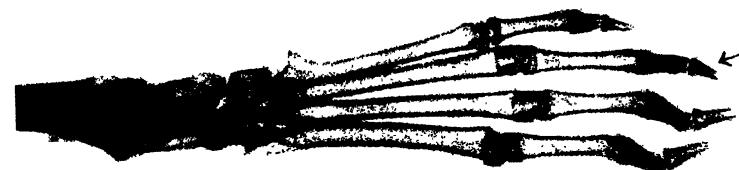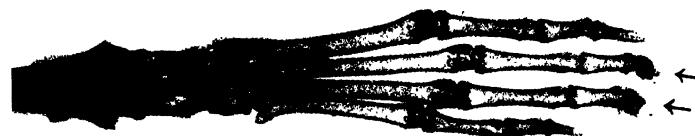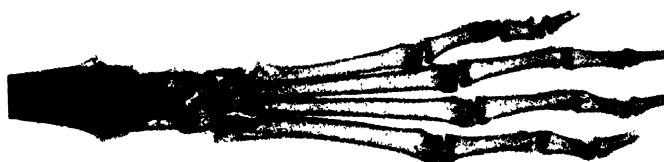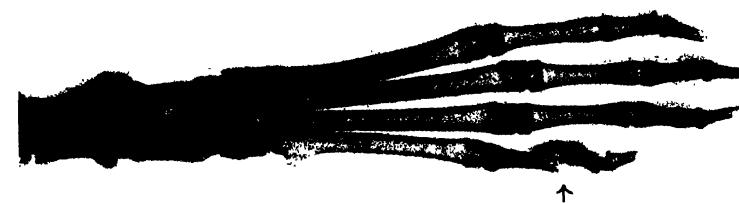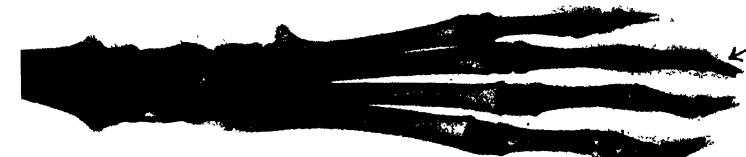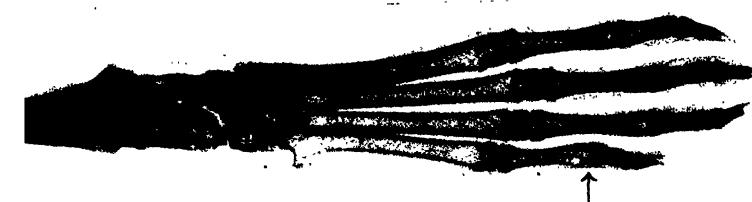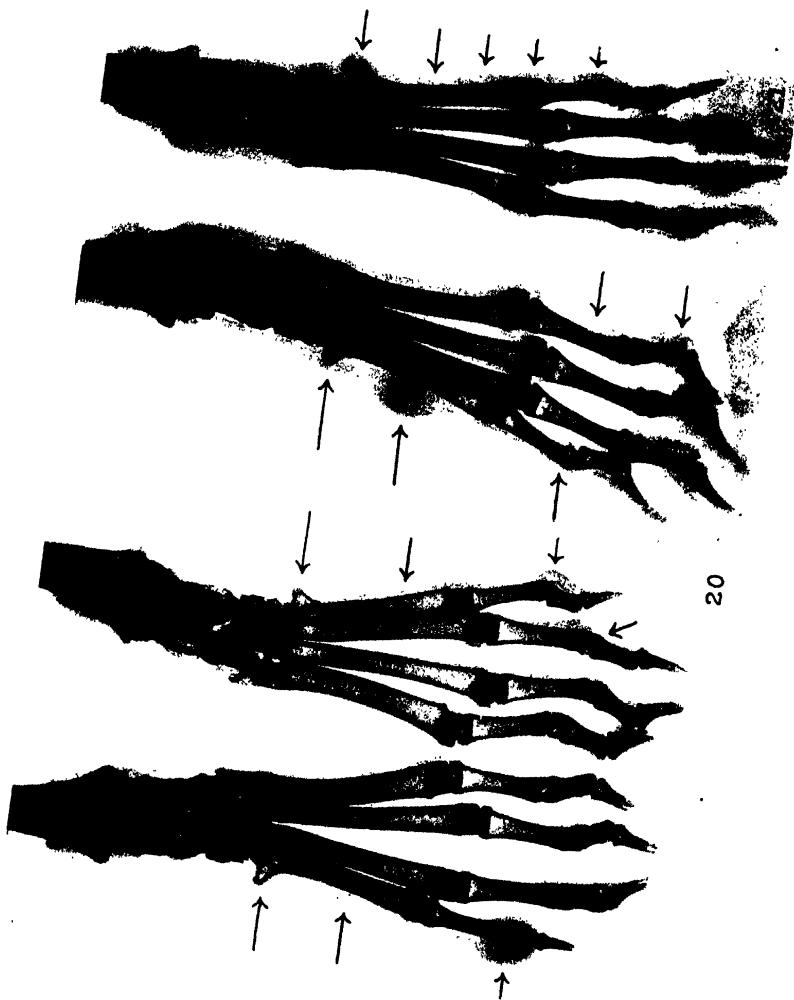
EXPERIMENTAL SYPHILIS IN THE RABBIT.

VII. AFFECTIONS OF THE EYES.

BY WADE H. BROWN, M.D., AND LOUISE PEARCE, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 7 TO 9.

(Received for publication, January 31, 1921.)

Infection of the eyes following inoculation of the genitalia was one of the first forms of generalized syphilis to be recognized in the rabbit and has been reported more often than any other metastatic condition. Our knowledge of these affections dates back to 1908–10 when the first cases of metastatic keratitis were reported by Grouven (1–3), Mezincescu (4), Uhlenhuth and Mulzer (5), and Truffi (6). Since that time, several forms of affections have been described. Those most frequently mentioned are conjunctivitis, pericorneal injection, and interstitial keratitis; of these, keratitis is perhaps the only one which possesses a clearly defined status.

In addition to this class of affections, lesions of the eye grounds including choroiditis and chorioretinitis were described by Nichols (7) and by Reasoner (8) working in collaboration with Green. With certain strains of *Treponema pallidum*, these conditions were noted in from 70 to 95 per cent of the animals examined (mode of inoculation not stated) which may indicate that lesions of the eye grounds are more common than those of other structures—at least with some strains of the organism.

Reasoner also reported an instance of cataract of both eyes and a gumma involving the ciliary body, the iris, and the lens.

Our own observations on affections of the eyes were confined to a study of those conditions that could be detected by the unaided eye and included, therefore, affections of only the more superficial structures such as the conjunctiva, the cornea, and the iris. This group of lesions has received more attention than any other class of syphilitic affections but there is still much which may be added to the facts

71

already recorded. In presenting the results of our observations, we shall not attempt to do more than describe changes which might be recognized by any one familiar with the simpler forms of eye lesions.

The difficulties of their sharp delimitation will doubtless be appreciated, and while the various groups of affections are separated from one another, as a matter of convenience in presentation, it must be understood that combination was one of the most characteristic features of eye involvement.

Pericorneal Injection.

Pericorneal or ciliary injection is a well recognized condition in rabbits infected with *Treponema pallidum* but in the literature it is practically always referred to in connection with keratitis and apparently no significance has been attached to it apart from this one condition. It is, however, the most frequent sign of infection of the eyes and was often noted in animals where no other gross abnormality could be detected as well as in association with affections of the conjunctiva, the cornea, and the iris.

It occurred in two fairly distinct forms. The one most often seen consisted of a ribbon-shaped band of distended vessels which extended from the fornix to the sclerocorneal margin (Fig. 1) where they spread out and partially encircled the cornea. The usual position of these vessels was the region of the superior rectus muscle but they were also seen in the lower or lateral sectors of the eye.

A second type of pericorneal injection, which is perhaps more analogous to that seen in the human eye, consisted of a series of prominent vessels which encircled the cornea, giving off a few small collaterals which radiated outward as shown in Figs. 2 and 3.

One of these forms appeared to be as characteristic as the other, and in the most pronounced cases, the two were combined in about equal proportion with zonal radiations extending outward from all parts of the cornea. This was the state of the eye in Fig. 1 which was a marked case of pericorneal injection associated with a moderate degree of acute conjunctivitis (shown on the upper lid), but as may be seen, there was no involvement of either the cornea or the iris. This condition lasted for only about 48 hours when the congestion subsided and the eye returned to normal.

Ciliary injection was the first sign of eye infection to appear and the history of a simple injection was usually that of a recurrent affection of short duration. During its early stages and in mild cases, it suggested no more than a vasomotor instability of some particular group of vessels which would suddenly become engorged and return to normal within a very short time, leaving some uncertainty as to whether any abnormality existed. Then a well established injection would take place and subside after a few days or the condition would gradually develop into an outspoken iritis or keratitis.

As yet, no attempt has been made to demonstrate spirochetes in a case of simple ciliary injection. Histologically, however, there were definite pathological changes. These consisted of vascular dilatation and engorgement of the vessels in the episcleral tissue and at the sclerocorneal junction; there were slight edema of these tissues and a moderate polyblastic infiltration which was chiefly perivascular. The identification of this affection as a manifestation of syphilis rests, therefore, upon clinical and histological evidence. A marked ciliary injection presents no difficulties of diagnosis, but unless the milder cases are followed by some more characteristic lesion, they cannot be recognized clinically as more than probable manifestations of syphilitic infection. The significant features are its transient character, the tendency to recurrent attacks, the frequency with which it eventually terminates in iritis or keratitis, and its constant association with these two conditions.

Conjunctivitis.

Inflammation of the conjunctiva was noted in connection with affections of both the nasolacrimal system and of the eye itself. In the latter instance, it usually preceded or was an accompaniment of other affections of the eye, and it was difficult to determine clinically whether the inflammation of the conjunctiva was the result of a conjunctival infection or only an associated manifestation of an infection centered elsewhere. There were instances, however, in which the conjunctiva appeared to be the chief or sole focus of infection, and, in any case, involvement of the eyes frequently gave rise to marked inflammatory reactions which might affect the entire conjunctiva or only certain limited areas.

An acute diffuse inflammatory reaction was frequently seen in association with a simple ciliary injection or during the early stages of a keratitis or iritis.   This form of affection began with reddening and swelling of the margins of the lids and conjunctiva, increased lacrimation, and drooping of the upper lids (Figs. 3, 11, 12, 17, and 19). It progressed rapidly and in some instances resulted in an intense congestion and swelling of the entire membrane with petechial hemorrhages and gray or yellowish gray patches scattered here and there.

In many instances, the inflammatory reaction was confined to a smaller area, the usual seat of such processes being the region of the superior rectus muscle or some part of the palpebral conjunctiva.

The acute manifestations were, as a rule, of short duration and disappeared completely within a few days.

No spirochetes were found in the lacrimal secretion of animals with this type of inflammatory reaction, and no effort was made to demonstrate them in the conjunctiva itself, but sections of the conjunctiva showed a characterisitc syphilitic infiltration.

Another form of conjunctival lesion which was frequently seen in rabbits infected with *Treponema pallidum* resembles somewhat the phlyctenular conjunctivitis of man.   These lesions were of three types, not all of which could be related to the syphilitic infection. In one group of cases, they appeared as distinct nodules or thickened patches situated at the sclerocorneal margin as in Figs. 4 and 5 or distributed along the course of the vessels in the superior quadrant of the eye (Fig. 6).   The nodules were of a pale, opalescent, or yellowish white color and were associated with a well marked ciliary injection as in Fig. 4 or with a vascular reaction which was confined to the affected part of the eye as in Fig. 5.

Usually, the nodules or patches remained comparatively small but in one instance a lesion of this type developed into a mass which practically filled the superior fornix and presented all of the characteristics of a syphilitic granuloma, as may be seen by reference to Fig. 6.

Dark-field examination of the lesions for spirochetes gave inconstant results.   Some of them showed spirochetes in abundance, while in others, no organisms were found.   The diagnosis of this group of

conditions was not difficult, however, since they were nearly always followed by or associated with characteristic lesions of the cornea or iris.

A second group of phlyctenular lesions differed from those described chiefly iñ that they were composed of small, ill defined, gelatinous masses of a pale pink or salmon color. Some of them presented much the same appearance as that in Fig. 5, while others showed no injection of the conjunctiva or episcleral tissues. An example of a lesion of this type is given in Fig. 7.

These affections were at times comparatively common in both infected and uninfected animals. No spirochetes have been found in them, but in some cases, the condition has progressed to a definite keratitis. In the absence of such a history, we know of no clinical means by which the etiology of lesions of this type can be determined.

There is also a third condition, analogous to those just described, which may be referred to briefly. The appearance presented is shown in Fig. 8. There were a series of small discrete nodules of a slightly translucent or opaque white color distributed along the sclerocorneal margin; there was no increased vascularity of the area, and in the few cases seen, the nodules remained essentially as they appear in this photograph. No spirochetes have been demonstrated in lesions of this type, and there is no evidence, either clinical or pathological, to indicate that they are of syphilitic origin, unless it be the fact that they are composed of masses of lymphoid cells which occupy the same position as analogous lesions of established syphilitic origin.

Keratitis.

Keratitis is the most easily recognized lesion of the eye and (with the possible exception of metastatic lesions of the testicle) has been reported more often than any other manifestation of a generalized infection. It might appear from this that keratitis is the most common of the generalized lesions but such is not necessarily the case; it may be either very common or very rare, depending upon a number of circumstances. As the infection is ordinarily propagated, however, keratitis is of very frequent occurrence with most strains of *Treponema pallidum.*

The form of keratitis usually observed in the rabbit is always preceded by a ciliary injection and frequently by a diffuse inflammatory reaction of the conjunctiva.   The lesion described by all writers is an interstitial or parenchymatous keratitis.   It is peripheral in origin, and while it may develop from any part of the cornea, or from its entire circumference, the most common location is the superior margin.

The corneal lesions appear either in the form of a delicate fringe of vessels which extend over the edge of the cornea or as a narrow zone of turbidity.   As the vascular network spreads, the cornea becomes clouded, or as the zone of infiltration extends centrally, it is followed by the development of a network of vessels so that in either case the lesion produced combines the two elements of corneal infiltration and pannus in a varying degree.   There are, therefore, three types of lesions: one, in which infiltration with consequent clouding and thickening of the cornea is especially marked, another in which the the vascular reaction and pannus are the most noticeable features, and a third which combines the two processes in about equal proportion.

Three early cases of the vascular and the infiltrative types of keratitis are illustrated in Figs. 9 to 12.   The eye in Fig. 9 shows a well marked ciliary injection with a delicate network of vessels extending over the cornea.   The area covered by this pannus was faintly clouded, but outside of this zone it was perfectly clear.   Fig. 10 shows a condition of essentially the same character except that in this case the lesion arose from the inferior margin of the corner.

In contrast to these, the eye in Fig. 11 shows a crescent-shaped area of slight opacity with a small opaque dot at its center (descemetitis) which extends from the limbus over the margin of the pupil.   At this time, the vessels were just beginning to appear at the corneal margin; 24 hours later (Fig. 12), the infiltration had increased very markedly, forming an opaque, elevated area with a narrow but dense pannus at its outer margin.   The relative proportion between the vascular and infiltrative reactions shown in these three cases was maintained throughout their development.

Attention may be called to the ciliary injection and inflammatory reaction in the conjunctiva associated with these, which is well shown in Figs. 11 and 12.

When fully developed, the picture presented in cases of keratitis was very variable. The classical conditions are those presented in Figs. 13 and 14 which show pronounced keratitis involving a large part of the cornea. The first of these (Fig. 13) shows three things: first, a diffuse clouding of the cornea extending well below the pupillary margin; second, dust-like particles of dense opacity which are best seen over the pupil; and third, a very marked and uniform pannus, which extends like a curtain over the upper portion of the cornea.

The second lesion is quite different from this. As in the preceding case, there is a milky clouding of the cornea with granular deposits on the posterior limiting membrane but the noticeable feature of difference is the absence of a well defined pannus or even of a marked pericorneal injection. There were, however, a few vascular filaments in the cornea and a faint pannus can be seen towards the anterior or internal angle of the eye. As seen in these two animals, descemetitis was usually present in cases of marked corneal involvement but was frequently absent in milder ones

Two older lesions of the cornea which will serve to complete the picture of this condition are reproduced in Figs. 15 and 16. The first of these affections (Fig. 15) was of 6 weeks duration and is given especially to illustrate the deep vascularization of the corneal lesion which frequently occurs during its later stages. The marked interstitial infiltration and descemetitis are quite obvious, and attention may be called also to the granulomatous lesion in the fornix.

The second lesion of this group (Fig. 16) had an unusual history of repeated relapses extending over a period of 27 months and is used to illustrate the formation of the salmon patch which is less constant in cases of keratitis in the rabbit than in man. The irregular area of dense opacity which extends from the upper margin of the cornea across the pupillary area developed 15 months after the first attack of keratitis. It was at first of a reddish gray color, subsequently changing to a pale orange, then yellow and gray. These appearances changed from time to time with the recurrent activity of the eye infection. Eventually the opacity diminished to a considerable extent, but a diffuse haziness of the cornea with a central opacity was still present when the animal was killed 27 months after the lesions first appeared.

Punctate areas of clouding or opacity, unaccompanied by a vascular reaction of any kind, have been observed in the central portion of the cornea in a few instances.   We have not been able to investigate these lesions, however, and nothing is known as to their cause or nature.

Briefly, therefore, the only known form of syphilic keratitis in the rabbit is an interstitial keratitis with pannus which usually arises from the superior margin of the cornea but may develop in other positions or as a circumcorneal affection.   Ordinarily only a portion of the cornea is involved and the alterations produced are relatively slight, but in more pronounced cases, the entire cornea may be affected, with the production of deep seated lesions which may persist for months or even years.

The process of resolution of corneal affections was an irregular one. Usually the infiltration was first to disappear, leaving a vascular network as the only mark of the previous lesion.   This is well brought out by a comparison of the eyes in Figs. 15 and 6, Fig. 6 representing a period 38 days later than Fig. 15.   The aberrant vessels were at times very slow to disappear and have been known to persist almost unchanged for several months.   In exceptional instances, the vessels disappeared before the infiltration, or resolution occurred in both directions at about the same rate.   As a rule, resolution was complete, leaving no mark of the previous lesion.

Iritis.

Apparently iritis has not been recognized as a manifestation of generalized syphilis in the rabbit distinct from that of the cornea, except in the one instance recorded by Reasoner of a gumma which involved the ciliary body, the iris, and the lens.   Among the animals studied by us, however, it was a very common affection.   Case records show a higher incidence of keratitis, but when the nature of the lesions and the relative ease or difficulty of their detection are considered, it appears quite likely that iritis was the more common of the two.   The majority of the cases were recorded in albino rabbits, and while we were inclined to attribute this to difficulties of diagnosis interposed by a pigmented iris, it must be recognized that the nature

of the iris may be an important factor in determining the occurrence of these lesions.

The conditions which we have classed as iritis varied from an acute hyperemia of relatively short duration to plastic and granulomatous processes which produced permanent lesions of the iris. While these affections are spoken of as iritis, it must be understood that in many cases the ciliary body as well as the iris was involved, but the exact location of the lesion could not be determined clinically so that the term iritis is used to cover a group of reactions which was characterized chiefly by readily recognizable changes in the iris. Microscopic examination of a number of eyes showed, however, that the lesion was at times practically confined to the base of the ciliary body, while in other cases the ciliary body, the iris, and the choroid were all involved.

The most common form of iritis was an acute diffuse affection which lasted for only a few days. The usual signs in these cases were photophobia and profuse lacrimation with conjunctival and ciliary injection which appeared before any definite alteration could be detected in the iris and lasted until the acute reaction began to abate. The appearance presented in such cases may be seen by comparing Figs. 17 and 18 which show the affected and the normal eyes of an animal at the very beginning of an attack of iritis and Figs. 19 and 20 which show respectively a slightly later stage of iritis and a photograph of the same eye taken 1 week later. (Both eyes of this animal were involved at the same time.)

The changes in the iris itself began with an acute hyperemia. In albino rabbits, the marginal and axial vessels stood out prominently, while the substance of the iris appeared but little altered (Figs. 17 and 21). As the condition progressed, the vessels became less conspicuous while the color of the iris deepened to a dull red, a cyanotic, or rose color (Figs. 17, 19, 21 and 22); occasionally small hemorrhages occurred (Fig. 22) and the structural details of the iris became blurred and indistinct (cf. Fig. 18 with Figs. 17 and 19 to 22).

This condition lasted for a very short time, as a rule (24 to 72 hours), the vascular dilatation then subsided, and the diffuse discoloration gradually disappeared, leaving the iris slightly more clouded than normal (cf. Figs. 18 and 20).

Meantime, certain alterations in the pupillary reaction were noted. Under the conditions of lighting used, the normal pupil measured from 7 to 9 mm. in diameter; with the development of an acute iritis, the pupil gradually contracted until it measured only 4 to 6 mm. in diameter (*cf.* figures) and reacted very sluggishly to light (flash). The pupil and the pupillary reflexes usually returned to normal as the iritis subsided.

In darkly pigmented irides not all of these changes could be detected. The conditions usually noted were a change in the color of the iris, clouding or opacity, and a narrowing of the pupil. These may be made out by comparing the infected and the normal eyes of the same animal in Figs. 23 and 24 respectively. In this instance, it can be seen that the iris of the infected eye is distinctly mottled and of a lighter color, as well as hazy, and that the pupil is narrower than that of the normal eye; this was the condition usually found in this class of animals.[1]

In the group of cases described, the contents of the anterior chamber were little if at all affected and there was no descemetitis. In a few instances of severe iritis, there were hemorrhages into the anterior chamber (see Fig. 16), or the aqueous was slightly clouded (Fig. 22) and there were granular deposits on the posterior limiting membrane; these are just visible in Fig. 23.

There were also a few cases of plastic iritis. These differed from the condition which has been described in the presence of a slight exudate, which appeared to form on the posterior surface of the iris and protruded beyond the pupillary margin as a grayish white or yellowish white film. In some instances, the pupil was contracted, irregular, and fixed, while in others it retained its normal shape and its accommodation to light was less impaired.

---

[1] In comparing the pupils and pupillary reactions of rabbits, it should be noted that in the ordinary diffuse light of the laboratory, there is a decided difference in the size and the accommodation to light between an albino rabbit and one with a dark colored iris. The pupil of the albino is relatively smaller and reacts quickly to light (flash), contracting to a very small diameter. The pupil of an animal with a dark colored iris reacts more slowly, and the extent of the contraction is much less—sometimes barely perceptible.

The inflammation in this group of cases also cleared up within a short period of time and the exudate was absorbed without the formation of fibrous synechias.

The diffuse congestive affections of the iris appeared to be referable to a lesion at the base of the ciliary body rather than of the iris itself. It was only in the more pronounced cases and in instances in which hemorrhage or exudative phenomena were present that definite lesions of the iris could be detected, and even in these the ciliary body appeared to be more affected than the iris, but this could not be determined clinically.

Diffuse affections of the iris merged by insensible degrees with another type of affection in which the main lesions were of a focal nature. Fewer of these have been seen and comparatively little is known of them. The most common condition observed was a focal area of congestion or hemorrhage, an example of which is given in Fig. 25. These lesions were single or multiple but produced no alteration in the iris except at the points involved, and symptoms of acute iritis were usually absent.

A second condition, which was more characteristic, is that shown in Fig. 26. These cases presented all of the manifestations of an acute diffuse iritis, but in addition, small granulomatous nodules developed in the substance of the iris, usually near the pupillary border. There was partial or complete fixation of the iris with some irregularity of the pupil.

The pendant nodule seen in Fig. 26 developed in the superior margin of the pupil but in some way became torn loose except at one point, leaving a wedge-shaped defect in the iris which is plainly visible. A second nodule is seen above and to the right of the point of attachment of the first.

As a rule, the granulomatous lesions were comparatively small and tended to resolve without the production of any marked injury to the iris. In one instance, however, a lesion of this type developed into a large granulomatous mass which involved a considerable portion of the iris and filled nearly half of the anterior chamber of the eye. This lesion is shown in Fig. 27.

The formation of posterior synechias resulting in permanent alterations of the pupil was very rare. In one animal of our series, this

condition developed very early and there was marked irregularity and almost complete occlusion of both pupils which lasted up to the time of the death of the animal—a period of nearly 2 years. The right eye of this animal is shown in Fig. 28. The dark area at the center of the eye represents the outline of the original pupil but at the time this photograph was taken, most of this area had been filled in with granulation tissue and the only aperture present was of very small size and can just be seen at the lower and anterior edge of the corneal opacity.

Clinical History.

Correlation of Eye Affections.—The clinical history of the eye affections described as pericorneal injections, conjunctivitis, keratitis, and iritis containsmany points of interest. They are so intimately related to one another that they can hardly be considered as entirely distinct forms of infection. One condition may occur without the others but the circumstances were such as to favor a combination of the various affections. Thus, pericorneal injection and conjunctivitis may occur in the absence of any definite lesion of the cornea or iris, but the latter conditions were always preceded or accompanied by pericorneal injection and conjunctivitis. In like manner, iritis and keratitis may occur independently, or they may occur together, or one condition may follow the other. This peculiar association rests upon a simple anatomical basis. Histologically, it was found that these affections could all be traced to a common lesion which was centered about the vessels which encircle the cornea. This lesion first makes its appearance in the wedge-shaped mass of loose connective tissue at the outer side of the sclerocorneal junction (episcleral tissues). The vessels in this area become dilated, the tissue is edematous and infiltrated with polyblasts. If the lesion is confined to this area, it manifests itself in the form of a ciliary injection. The infection tends to spread, however, extending to the conjunctiva, to the substance of the cornea, or inward towards the canal of Schlemm and the spaces of Fontana. This led to the development of lesions in the cornea, the ciliary body, the iris, and occasionally in the choroid, giving rise to clinical signs of infection, the nature of which depended upon the parts thus involved.

When viewed from this standpoint, the eye affections described assume a less complex character.

Predisposing Factors and the Relation to Other Manifestations of Infection.—As was mentioned in connection with keratitis, affections of the eyes may be very frequent or very rare with a given strain of *Treponema pallidum*, depending upon a variety of conditions. From an analysis of the circumstances under which eye infections occurred in our series of animals, it was found that in more than 75 per cent of the cases, they were the only generalized lesions which occurred or were the last type of lesion to appear. In other words, eye lesions appeared to occupy a terminal position in the sequence of tissue reactions.

It was also found that experimental conditions could be employed which would emphasize this relation. These cannot be discussed further than to say that in general, circumstances which were unfavorable for the occurrence of other generalized lesions, tended to increase the relative incidence of affections of the eyes and conversely, those conditions which were most favorable for the occurrence of other lesions tended to reduce the relative incidence of eye lesions. Thus, double inoculation and late castration produced a high percentage of eye affections while unilateral inoculation and early castration reduced the incidence of these lesions.

Occurrence and Duration.—Considered collectively, eye lesions occurred at about the same interval of time after inoculation as other generalized lesions. The first lesions usually appeared at from 2 to 3 months after inoculation. Pericorneal injection and conjunctivitis were among the early affections, while keratitis and iritis occurred slightly later on the whole and the majority of the cases appeared near the end of the 3 months period. In several instances, eye lesions did not appear for as much as 6 to 8 months after inocculation, and in one animal, the interval was 2 years and 3 months, and in another, approximately 3 years.

One or both eyes might be involved, either simultaneously or in rapid succession. Less often, there was a considerable interval between the appearance of the two lesions. The duration of the affection was very short as a rule; simple pericorneal injections, diffuse conjunctivitis, and iritis frequently lasted for only a few days but

were of longer duration when complicated by lesions of the cornea. Keratitis was the most enduring, but even this rarely lasted longer than 2 to 3 weeks. The most marked corneal lesions lasted for several months, and in the one instance described above the lesions had not cleared up when the animal was killed 33 months after inoculation.

Recurrence.—Exacerbation of partially resolved lesions of the eyes or recurrence of completely healed lesions was almost the rule among those animals which were held over long periods of time. Two or three attacks of iritis and especially of keratitis were quite common. In one animal, numerous attacks of keratitis and iritis occurred over a period of approximately 2 years. Actual count of the attacks was lost but the number may be safely placed at more than a dozen. Several additional instances were recorded in which recurrent lesions appeared more than a year after inoculation.

Relapse of partially or completely healed lesions of the eyes occurred more often than in any other class of affections. The tendency to relapse in the case of keratitis has been noted by numerous observers and is the one redux phenomenon of the experimental infection which has received general recognition.

The tendency of eye lesions to relapse as well as the peculiar circumstances of their occurrence is probably to be explained by the degree of protection afforded these parts by reactions elsewhere and the feeble protection afforded by the reaction developed in these tissues.

SUMMARY AND CONCLUSIONS.

From the study of a number of instances of eye infection in the rabbit, it was found that a variety of affections might occur following scrotal or testicular inoculations of *Treponema pallidum*. Those observed included ciliary injection, conjunctivitis, keratitis, and iritis which might occur separately or in combination with one another, except that keratitis and iritis were always accompanied by a reaction in the ciliary vessels and usually by a conjunctivitis.

Several forms of each of these affections were described, and while some of them were regarded as presenting a very characteristic picture, it was recognized that the conditions present in other cases

were not sufficiently distinctive to permit of a clinical diagnosis. With a few exceptions, however, the pathology of the lesions was sufficient to identify them as processes of a syphilitic nature.

It was also found that this group of lesions usually arose from a common focus of infection which was located in the episcleral tissues immediately surrounding the cornea. From this point, the infection tended to spread to the conjunctiva and the cornea, or toward the canal of Schlemm and the spaces of Fontana and thence to the ciliary body, the iris, and the choroid. The localization of the lesion and the mode of extension were held to be responsible for the combination of manifestations usually observed.

From an analysis of the circumstances under which affections of the eyes occurred, it was found that the great majority of them occupied a definite position in the scheme of tissue reactions, being the only generalized lesions developed or the last type of lesion to appear.

These facts, together with the unusual frequency of relapse in these affections, were believed to indicate that a low degree of protection was conferred upon these tissues by reactions taking place elsewhere and that the protection afforded by the local reaction was of a feeble character. This deduction was in part confirmed by the fact that it was found to be possible to increase or decrease the incidence of eye lesions by the use of experimental means which varied the scheme of reaction in animals inoculated with a given strain of *Treponema pallidum.*

BIBLIOGRAPHY.

1. Grouven, C., *Dermat. Z.*, 1908, xv, 209.
2. Grouven, C., *Verhandl. deutsch. dermat. Ges., X. Kong.*, 1908, x, 212.
3. Grouven, C., *Dermat. Z.*, 1910, xvii, 161.
4. Mezincescu, D., *Deutsch. med. Woch.*, 1909, xxxv, 1188.
5. Uhlenhuth and Mulzer, *Arb. k. Gsndhtsamte*, 1913, xliv, 307. (See p. 337.)
6. Truffi, M., *Centr. Bakt., 1te Abt., Orig.*, 1909, lii, 555.
7. Nichols, H. J., *J. Exp. Med.*, 1914, xix, 362.
8. Reasoner, M. A., *J. Am. Med. Assn.*, 1916, lxvii, 1799.

EXPLANATION OF PLATES.

The illustrations are reproductions of photographs which represent the objects at their natural size. Unless otherwise indicated, statements of time are estimated from the date of inoculation.

PLATE 7.

FIGS. 1 to 8. Ciliary injection, and affections of the conjunctiva and episcleral tissues.

FIG. 1. 61 days. A typical example of ciliary injection with acute conjunctivitis.

FIG. 2. 88 days. Circumcorneal injection (ciliary) with acute conjunctivitis.

FIG. 3. 132 days. Acute diffuse conjunctivitis with pronounced swelling and intense redness of the conjunctiva. There is also a moderate pericorneal injection.

FIG. 4. 54 days. Miliary granulomata of the pericorneal tissues associated with ciliary injection.

FIG. 5. 64 days. A phlyctenular lesion of the conjunctiva and episcleral tissues.

FIG. 6. 156 days. A syphilitic granuloma involving the conjunctiva and episclera. There is also an old lesion of the cornea which is undergoing resolution.

FIG. 7. 45 days. A pericorneal nodule of a type which may or may not be syphilitic. Note the absence of a vascular reaction.

FIG. 8. 49 days. Multiple lymphoid nodules in the pericorneal tissues which apparently are not of syphilitic origin.

PLATE 8.

FIGS. 9 to 16. Lesions of the cornea.

FIG. 9. 54 days. Interstitial keratitis. Ciliary injection and early vascularization of the cornea.

FIG. 10. 100 days. An early keratitis of the lower margin of the cornea analogous to that in Fig. 9.

FIG. 11. 95 days. An early keratitis of the infiltrative type. There are a well marked pericorneal injection and conjunctivitis with a slight descemetitis.

FIG. 12. 24 hours later. There are an increase in the inflammatory reaction and a well developed pannus at the margin of the cornea.

FIG. 13. 73 days. A typical example of interstitial keratitis with marked pannus.

FIG. 14. 106 days. An example of interstitial keratitis showing marked infiltration of the cornea with comparatively slight vascularization.

FIG. 15. 133 days. Interstitial keratitis involving the entire cornea with deep vascularization.

FIG. 16. 1 year, 8½ months. Keratitis with well marked salmon patch covering large area of cornea. The dark spot in the eye is due to hemorrhage in the iris.

PLATE 9.

FIGS. 17 to 28. Affections of the iris.

FIG. 17. 94 days. Acute iritis showing drooping of the upper lid, slight lacrimation, and slight contraction of the pupil.

FIG. 18. 94 days. Normal eye of the same ainmal as that in Fig. 17 given for comparison.

FIG. 19. 70 days. Acute iritis, partial closure of the lids, and lacrimation. Narrowing of the pupil and clouding of the iris.

FIG. 20. 1 week later. Same eye as that in Fig. 19. Photograph shows partial recovery from the iritis. Eye appears normal except for the loss of structure in the iris. *Cf.* with Fig. 18.

FIG. 21. 132 days. An early acute diffuse iritis. There is a well marked pericorneal injection. Vessels of the iris are engorged and its substance is clouded. There is also clouding of the contents of the anterior chamber. The right eye of this animal is shown in Fig. 3.

FIG. 22. 125 days. Acute diffuse iritis, or slightly later stage than that shown in Fig. 21. Vessels are no longer visible but the iris is distinctly clouded and there is a focus of hemorrhage immediately above the pupil.

FIG. 23. 85 days. Acute iritis in an animal with pigmented iris. *Cf.* with Fig. 24. The iris is of a lighter color than normal, somewhat mottled and the structure indistinct. Pupil is also contracted.

FIG. 24. 85 days. Normal eye of the same animal as that in Fig. 23.

FIG. 25. 95 days. Focal lesion of the iris. Remainder of the iris appears entirely normal.

FIG. 26. 82 days. Acute diffuse iritis with a granuloma, superior margin of the pupil. The small nodule seen at the upper margin of the iris has been torn loose from its original position, leaving defect in the margin of the pupil.

FIG. 27. 185 days. Large granulomatous lesion of the iris projecting into the anterior chamber of the eye.

FIG. 28. 1 year, 7 months. Occlusion of the pupil with irregularity and fixation. Permanent lesion.
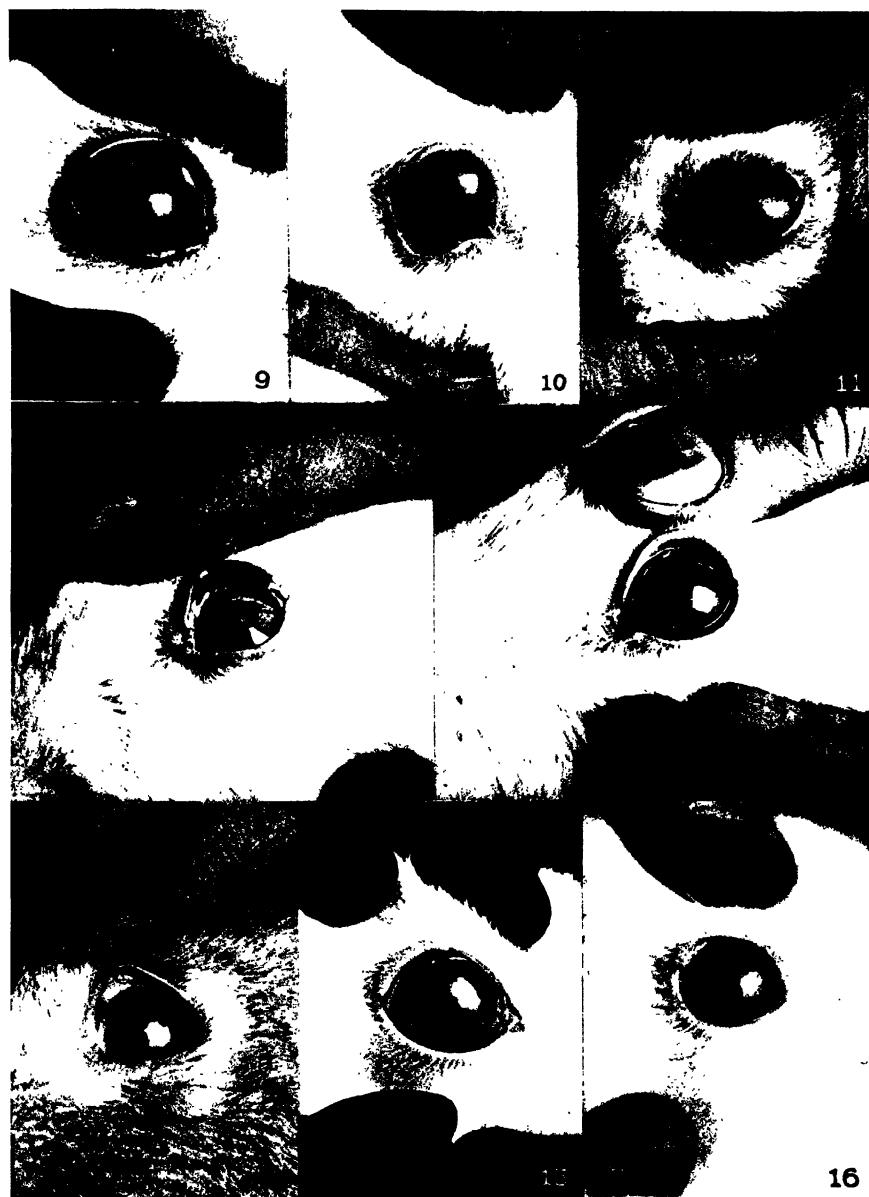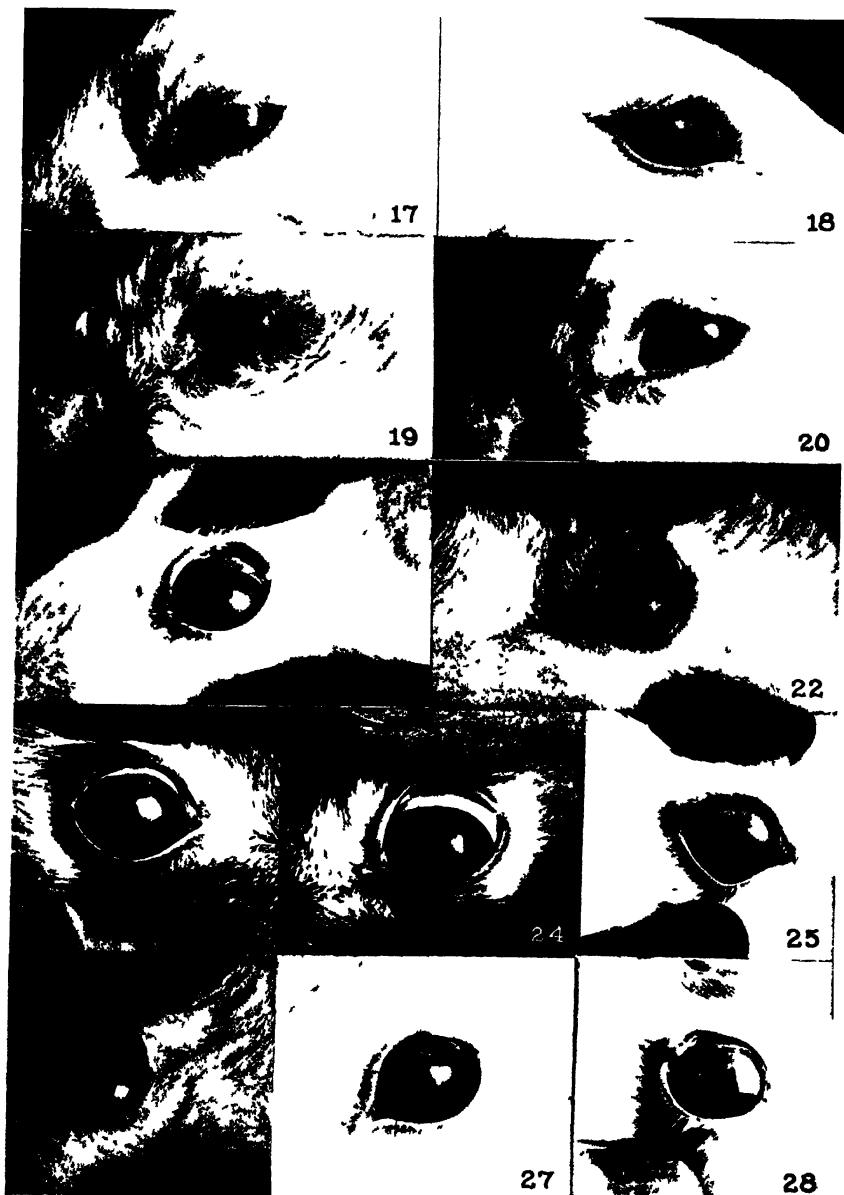
SUPERINFECTION IN EXPERIMENTAL SYPHILIS FOL-LOWING THE ADMINISTRATION OF SUBCURATIVE DOSES OF ARSPHENAMINE OR NEOARSPHENAMINE.*

BY WADE H. BROWN, M.D., AND LOUISE PEARCE, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 73 AND 74.

It is generally held that a person infected with the virus of syphilis becomes practically immune to a second infection—that, with the development of the initial lesion, a condition becomes established which makes it difficult or impossible to superimpose a second infec-tion upon the one already present and that this refractory state is maintained as long as an infection exists. As far as they have been tested, human and animal experiences are in essential agreement upon these points.

In the absence of any evidence to the contrary, it has been assumed that the principles contained in this conception of syphilitic immunity apply to treated as well as to untreated cases of infection and many syphilographers have regarded the appearance of fresh lesions of the chancre type, under circumstances which would indicate a new infec-tion, as the most conclusive evidence of the cure of the previous infection. There are some, however, who have questioned the valid-ity of so called reinfections, and others who have objected to their interpretation as evidence of cure. Granting that the class of cases referred to represents instances of true, second infection, an alterna-tive interpretation of such occurrences has recently been presented by Jacobi (1) in which it is suggested that the reaction to a second inoculation may be viewed more as an expression of an existing state of immunity than as evidence of the presence or absence of infection.

The situation which exists with reference to the immunity of infected individuals, the possibilities of superinfection or reinfection, and hence the interpretation to be placed upon the appearance of manifestations of disease which give every evidence of being due to a new infection are both complicated and obscure.   Until the introduction of modern methods of spirocheticidal therapy, instances of so called reinfection were comparatively rare, and interest in the subject was largely a theoretical one.   Within the past few years, however, the literature has contained numerous reports of reinfection following treatment with arsphenamine or neoarsphenamine, and the interpretation to be placed upon these occurrences has become a matter of immediate practical importance.   Where the evidence of a new infection seemed sufficient, the general tendency has been to accept such infections as proof of a cure, and this would appear to be the logical interpretation unless it can be shown that under circumstances such as have existed in these cases, superinfection becomes possible.   An element of uncertainty is introduced by the treatment employed, since practically nothing is known concerning the influence of such drugs as arsphenamine and neoarsphenamine upon syphilitic immunity.

The point at issue, therefore, is not so much a question of the immunity conferred by syphilitic infection as it is the effect which given therapeutic agents may have upon the resistance of infected individuals, whether native or acquired, and upon any spirochetes which may survive their action.   Presented in this form, the problem of reinfection may be approached experimentally as one of the influence of drug action upon immunity and infection, and experiments have been carried out in animals for the purpose of determining the effect of subcurative doses of arsphenamine and of neoarsphenamine upon the resistance of infected animals to reinoculation.   These experiments were divided into two groups according to the stage or progress of the infection, and the work to be reported in this paper deals with the results of treatment and reinoculation of animals with early but marked primary lesions.

EXPERIMENTAL.

The experiments to be reported consisted of the infection and treatment of two sets of rabbits—one with arsphenamine and the other with neoarsphenamine—after which they were reinoculated for the

purpose of determining their susceptibility to a new infection as indicated by the production of lesions at the site of inoculation. The results of the experiments were controlled in four ways, (1) as to the effects of the treatment employed, (2) as to the immunity developed by the infection, (3) as to the relative susceptibility of normal animals as indicated by their reaction to the virus used in the reinoculation experiments, and (4) by the use of a virus of essentially the same virulence as that causing the existing infection.

All animals were kept under observation for a minimum of 8 weeks after treatment while the therapeutic controls were held for 4 months as a means of affording a more accurate estimation of the status of the infection existing after treatment.

Infection of the Animals Used.—The animals used were inoculated in both testicles with 0.2 cc. of an emulsion containing one to three spirochetes to the microscopic field (Nichols strain). The object in view was the production of an intense infection which would confer a high degree of protection against reinoculation within a short period of time. The incubation period of the testicular lesions averaged about 10 days and the lesions developed very rapidly; at the end of 18 days the testicles were markedly enlarged and indurated, and in some instances there was an edema of the scrotum, indicating that the lesions were approaching the height of their first cycle of development. The animals were then divided into three groups according to the degree of development of the testicular lesions: Five animals with the most advanced infections were placed in the group to be treated and reinoculated; five others with the least advanced lesions were set aside as infected controls to be reinoculated at the same time as the treated animals; while a third group, consisting of animals showing various degrees of testicular involvement, were treated as therapeutic controls for the reinoculated animals of the first group.

Treatment.—Treatment was carried out 18 days after inoculation by the intravenous administration of a single dose of arsphenamine or neoarsphenamine. The products used were of the original German manufacture, being from Lots A 25819 and A 25884 respectively. The arsphenamine was neutralized by the addition of the theoretical amount of N sodium hydroxide to form the disodium salt, and both substances were administered in a 0.2 per cent solution.

The drugs were employed in the equivalent amounts as stated by the manufacturer. The doses used were 6 mg. of arsphenamine per kilo of body weight and 9 mg. of neoarsphenamine. The selection of these doses was based upon known values of therapeutic action for arsphenamine rather than neoarsphenamine and represented an attempt to use a dose of this drug which in the average animal of the group would yield a therapeutic effect of a definite character; namely, a regression of lesions approximating complete resolution with freedom from recurrence for 4 to 6 weeks followed by clinical relapse within a period of not more than 2 to 3 months. The ability to gauge these effects correctly was considered to be one of the two most essential features of the experiments, the other being reinoculation.

Twelve rabbits were treated with each of the drugs in the manner described; five of them were subsequently reinoculated and the seven others held as therapeutic controls.

Reinoculation.—Reinoculation of treated and untreated animals was carried out on the 24th day of the infection (5 days after treatment). In order that there might be as little difference as possible in the virulence of the organisms used for reinoculation and those producing the original infection, the virus used was obtained from an animal of the same series as those to be reinoculated. This was, in a sense, a measure of control. Each animal received an intracutaneous injection of 0.2 cc. of an emulsion containing one to three spirochetes to the microscopic field at two widely separated points, the ventral surface of the sheath and the base of the right ear in the region of the posterior marginal vein (see illustrations). It will be noted that one of these points was in close proximity to the original lesions and the other as far removed as was practicable. This choice of sites for reinoculation was determined in part by the susceptibility of skin areas and in part was used as a means of checking the extension of the immunity in control animals.

Reinoculations were timed both with reference to the possible retention of drug in a biologically active state and with reference to the progress of drug effects, the intention being to reinoculate as early as possible so as to give the best opportunity for the development of lesions before recurrence of the original lesions took place.

Methods of Control.—The results of the experiments outlined were controlled in four ways:

1. Therapeutic Controls.—In order to avoid any confusion which might arise from an attempt to interpret therapeutic effects obtained in animals which had been reinoculated, seven rabbits from each of the treated groups were held under observation for the purpose of determining as nearly as possible the effect which had been produced upon the original infection by the treatment employed.

2. Infected Controls.—Five infected rabbits of the same series as those used for treatment were reinoculated in the manner described with the same material as that used for reinoculation of the treated animals and for the inoculation of the normal controls.

3. Normal Controls.—The relative susceptibility of normal animals to the virus used for reinoculation was controlled by the inoculation of three normal rabbits carried out in the same manner as that of treated animals. These will be referred to as normal controls.

4. Virus Control.—As a means of insuring equality in the virulence of the spirochetes originally introduced and those used for the second inoculation, the reinoculations were made with material taken from a testicle of an animal of the same series as those to be reinoculated.

Effects of Treatment.

A consideration of the results obtained from the experiments outlined above should logically begin with the effects of the treatment employed. Following administration of the drugs, the testicular lesions began to regress, resolution proceeding somewhat more rapidly in the animals treated with neoarsphenamine than in those with arsphenamine. In some animals, the lesions disappeared completely by the end of 7 to 14 days, at which time the effect of the drug upon existing lesions practically ceased.

Of the twelve animals treated with neoarsphenamine, the testicular lesions were completely resolved in seven, four showed definite residual lesions in the form of diffuse thickenings or of circumscribed nodules, and in one the result was uncertain. In contrast to this, complete resolution occurred in only four of the animals treated with arsphenamine—a difference which is quite characteristic of the action of the two drugs in inducing resolution or healing of lesions in the experimental animal.

The first evidences of relapse among the therapeutic controls were noted between 14 and 17 days after treatment. Four of the seven animals treated with arsphenamine showed reinduration and gradual increase in the size of residual lesions or the development of new foci of infection by the end of the 3rd week, and the infection progressed at a normal rate. A fifth animal relapsed 45 days after treatment, while the condition in the two others was not clear. From the 3rd week onward, there were brief periods during which the testicles of these two animals appeared to enlarge somewhat, there were ill defined areas of thickening, and even a few tiny nodules in the testicles or tunics, all of which were suggestive of relapse, but none of these conditions developed into affections which exhibited the characteristic clinical appearance of syphilitic lesions. At the end of 3 months, test inoculations were made from popliteal lymph nodes of both animals with positive results, and one of them developed a testicular lesion shortly afterwards.

Clinical relapse among the animals treated with neoarsphenamine was more delayed. Three animals of the control group showed minor changes suggestive of relapse as early as 14 days after treatment, but outspoken lesions did not develop until towards the end of the 3rd month. Relapse occurred in the four others between the 24th and 39th days, but in two of these the growth of the lesions was again rather slow and irregular for from 4 to 6 weeks.

Infection was, therefore, shown to be present in all the therapeutic controls of both series. In other words, none of the animals were cured by the treatment received, and similar but less marked effects were produced by the treatment given the animals which were used for reinoculation.

Results from Reinoculation of Infected Controls.

The second point to be considered is the probable state of immunity which had developed in the treated and reinoculated animals. It was not possible to determine this with certainty, and in reality such a determination was not essential to the object of these experiments. However, as the best means of estimating the immunity in these rabbits and of controlling the results of reinoculation at the same time,

which was of more importance, animals with the most advanced infections were selected for treatment, while those showing the least progress of the infection were used as controls. In this way, the state of the infection existing at the time of reinoculation of the untreated animals was practically the same as that which had existed at the time of treatment of the other group.

The results from reinoculation of the infected controls may be given briefly. Within a few hours, all animals of the series showed a slight acute reaction at the site of inoculation which consisted first of an edema and then of a slight diffuse redness about the site of inoculation. This reaction subsided completely within 24 to 48 hours and in one animal was the only reaction observed. In the others, a diffuse or papular infiltration developed in the sheath and at the base of the ear. The lesions in the sheath reached their height in from 5 to 7 days and disappeared completely within 12 to 14 days. The papules measured from 3 to 6 mm. in cross-diameter; they were of a rose-pink color, firmly indurated, and of a slightly translucent appearance. The more diffuse lesions presented essentially the same characteristics. The ear lesions developed somewhat more slowly, and in three of the five animals were very slight, diffuse infiltrations lasting approximately 3 weeks. The majority of the lesions described were of the type of slight non-specific inflammatory processes, or they might be regarded as allergic reactions. It is possible that some of them were due to a slight but transient local infection, but no examination was made for spirochetes for fear that the trauma inflicted might induce regression.[1]

In two instances out of the ten inoculations (two injections in each animal), small, firmly indurated, and translucent papules developed at the base of the ear (Figs. 1 and 2). Clinically, the lesions presented the appearance of syphilitic granulomata. One of them disappeared within 3 weeks, and while the other never developed to more than 4 to 6 mm. in diameter, it persisted for 54 days.

These were the only instances in which reinoculation resulted in the production of skin lesions which gave evidence of being due to

---

[1] While trauma in some form appears to play a part in the distribution and even in the development of syphilitic lesions in the rabbit, scarification, cutting, or aspiration with a needle frequently causes them to regress.

infection, and they are reproduced in Figs. 1 and 2 for purposes of comparison with the lesions obtained by reinoculation of the treated animals. It is to be noted that both lesions occurred on the ear, while the foreskin was entirely negative, which is the reverse of the order of susceptibility of the two skin areas in normal rabbits.

Results from Reinoculation of Treated Animals and Normal Controls.

Reinoculation of treated animals gave results which were strikingly different from those of the infected controls. In a word, all except two of them developed perfectly typical chancres, examples of which are given in Figs. 3 to 12; there was marked lymphadenitis such as is associated with primary lesions, and spirochetes were present in abundance. Within 7 days, every animal of the group showed a characteristic syphilitic reaction at the site of inoculation, either in the form of an elevated papule or of a flattened area of infiltration. Of the twenty points inoculated, nineteen were positive by the 7th day, and a lesion appeared at the one remaining focus on the 11th day after inoculation. The incubation periods of these lesions coincided with those of the normal controls.

The growth of both the sheath and ear lesions in four of the ten animals, including two treated with arsphenamine (Figs. 3 to 7) and two with neoarsphenamine (Figs. 8 to 12), was extremely rapid and practically uninterrupted until they reached the stages of development shown in Figs. 3 to 12, and some of them progressed beyond the points shown. On the other hand, none of the three normal controls developed lesions which were at all comparable to the sheath and ear lesions of these four treated animals.

In another animal (arsphenamine), the lesion on the sheath grew somewhat irregularly, but within 4 weeks formed a characteristic ulcerated chancre measuring 1 cm. in diameter. Growth then ceased for a short time, but the lesion was considerably larger and increasing actively when the animal was discarded. The ear lesion was of the nature of a papule surrounded by a zone of diffuse infiltration. It developed to approximately 8 mm. in diameter during the first 4 weeks but had practically disappeared before the animal was discarded.

The lesions of a sixth animal (neoarsphenamine) grew more rapidly than those of any other for about 3 weeks, but development ceased

at this point. On the sheath, there was a lenticular lesion measuring 5 to 7 mm. in thickness at its center and spreading diffusely over an area more than 1 cm. in diameter. A similar but less elevated lesion developed at the base of the ear, and while there was some exfoliation over the center of both lesions, neither of them underwent ulceration. They gradually subsided and had practically disappeared at the end of the 2 month period of observation. It is noteworthy that this animal developed a slight periostitis of the nasal bones 39 days after treatment and later a lesion of the cornea. There was also a marked popliteal lymphadenitis such as is commonly associated with focal infections in the drainage area.

In two other animals of the arsphenamine group, the reaction during the first few weeks after reinoculation was comparatively slight. During the 4th week, however, both the ear and sheath lesions of one animal began to increase rapidly and developed into characteristic chancre-like lesions of approximately 1 cm. in cross-diameter. The ear lesion of the other animal was first a papule, then a diffuse infiltration, but a typical chancre measuring 8 mm. in diameter developed on the sheath.

The lesions produced in the two remaining animals of the treated and reinoculated series were comparatively slight and consisted of small papules or diffuse infiltrations. They were more pronounced and more enduring than any lesion of the infected controls but less than those of the normal controls. The therapeutic response in one of these animals, both of which were treated with neoarsphenamine, was apparently less than that in any other animal of the series. The original lesions were never completely resolved, and a clinical relapse was recognized 17 days after treatment.

A feature of especial interest in these experiments was the fact that relapse of the original lesions occurred in eight of the ten reinoculated animals at about the time the superinfection lesions became well established. In most instances, the second chancres overgrew (inhibited) the recurrent lesions, but in other animals, both sets of lesions developed together.

An excellent illustration of this phenomenon of the double infection is furnished by Figs. 6 and 7. Inspection of Fig. 6 will show that there is a diffuse enlargement of the right testicle and two large nodules are well outlined at the positions indicated by the arrows. There is also a small patch of infiltration in

the left scrotum. The nodules in the testicle existed from the time of treatment, and although they were considerably softened and diminished in size, they became reindurated and began to enlarge by the end of the 2nd week. The development attained within 5 weeks (the time represented by this photograph) would of itself preclude the possibility of a metastatic origin. The further development of the two sets of lesions is shown in Fig. 7.

The results of the treatment and reinoculation experiments as a whole may be stated as follows: Of the five animals treated with arsphenamine, and then reinoculated, the original lesions were completely resolved in only one instance and relapse occurred within 33 days in four of the five animals, including the one animal whose lesions had been resolved. The other animal of the group showed no definite increase in the testicular lesions during the period of observation, and the lesions which resulted from reinoculation were less marked than those of the other animals. By reinoculation, characteristic chancres were produced in all the animals of the group. Three of the five animals developed well marked lesions at the base of the ear as well as on the sheath, while the ear lesions were comparatively slight in the two others.

The results after treatment with neoarsphenamine were not so uniform. The testicular lesions were quickly resolved in two of the five animals. The testicles of a third animal were left slightly enlarged and diffusely thickened, but no further change occurred, either in the way of regression or progression of the lesions during the period of observation, and while well marked lesions were produced by reinoculation on both the ear and the sheath, they were of shorter duration than those of the other animals. Clinical relapse occurred in all other animals of the group at from 14 to 24 days after treatment. Characteristic chancres were obtained from the second inoculation in three of the five animals on both the sheath and ear; the other two gave only diffuse or papular infiltrations.

The three normal controls developed characteristic chancres on the sheath which measured from 1 to 1.5 cm. in diameter. One of them developed a large nodule in the subcutaneous tissues at the base of the ear in addition to a diffuse infiltration of the skin and the usual lymphadenitis. In the two others, there were small papules surrounded by a zone of diffuse infiltration, but none of them developed chancre-like lesions in this location.

DISCUSSION.

In attempting to give an interpretation of the experiments reported, the first point to be considered is that while one group of animals with well developed primary lesions of the testicles proved to be extremely refractory to a second cutaneous inoculation with a virus of equivalent virulence, a second group of animals from the same series, after treatment with arsphenamine or neoarsphenamine, was highly susceptible and with two exceptions reacted to the second inoculation with the development of characteristic manifestations of a primary infection. In fact, if susceptibility may be gauged by the reaction at the site of inoculation, some of the treated animals (four out of ten) were even more susceptible to infection than the normal controls. Disregarding for the moment the therapeutic result in as far as the original infection is concerned, it is certain that this difference in the reaction of the treated and untreated animals can be attributed to no other cause than the treatment employed.

In the second place, it is practically certain that none of the animals was cured. In the majority of them, the original lesions were not completely resolved, but there still might be some doubt as to whether the relapses which occurred in the reinoculated animals were true relapses of the original infection or were lesions arising from the second inoculation. In several instances, the clinical history of the relapse appeared to be sufficient in itself to exclude the latter possibility, since within a time too short to permit of the development of metastatic lesions, reinduration and growth occurred in existing lesions. In order to remove any possible confusion which might arise from this source, however, fourteen therapeutic controls were set aside—infected and treated with the same material and in the same manner as the reinoculated animals—and none of these was cured.

The results of the experiments may, therefore, be reduced to a very simple statement; namely, that treatment of animals with marked primary lesions of the testicles altered their resistance to such an extent as to render them susceptible to a second cutaneous infection without having effected a cure of the original infection. It is clear, therefore, that under the circumstances existing in these experiments, not only is superinfection of rabbits possible but animals treated in the manner

described may be rendered even more subject to a new infection than a normal animal.

It should be emphasized that these findings do not conflict in any way with established facts of syphilitic immunity. The conflict, if there is one, is with the assumption that the same conditions obtain in treated as in untreated infections or that an immunity once established cannot be altered.

It has been clearly shown by the work of Finger and Landsteiner (2) and of others that immunity to a second infection is not always absolute—that by resorting to the use of large doses of virus, it is possible, even in advanced cases of syphilis, to produce superinfection, or lesions at the site of inoculation which tend to assume the form of those characteristic of the stage of infection during which reinocula-lation is carried out. It is also well known that superinfection with the production of typical chancres is comparatively easy during the first incubation period, and multiple chancres and autoinoculation with chancre-like lesions are explainable upon this basis.

The facts enumerated have been found to apply to both human and animal infections. From the data available, the chief differences which appear to exist in untreated infections are that protection against reinfection develops much more quickly, is more marked, and more enduring in the rabbit than in man. These few facts furnish all the basis necessary for an understanding of the phenomena of a second infection following treatment which is not curative. It is only necessary to consider that treatment with such substances as arsphenamine and neoarsphenamine may cause an infection to revert to the condition which existed during the first incubation period when spirochetes were present but no immunity had developed; further, that the spirochetes which survive the action of the drug employed may be so attenuated or enfeebled for the time being as to be incapable of arousing an antagonistic reaction on the part of the host, thus favoring the propagation of more vigorous organisms introduced from without and the production of characteristic primary lesions at the site of the new infection.

These were the postulates which formed the basis for the experiments reported, and the results would appear to justify the conclusion that, in as far as early infections of the rabbit are concerned,

treatment with arsphenamine or neoarsphenamine may alter the immunological status of the animal to such an extent as to favor the propagation of a second infection without having accomplished a cure of the first. In advanced infections, conditions are more complicated, and it is more difficult to obtain chancres from second inoculations, but experiments now in progress indicate that even here superinfection is still possible of attainment.

Apart from any bearing which these experiments may have upon problems of syphilitic immunity, they serve to emphasize the necessity for a careful consideration of the influence which any therapeutic agent or any system of therapy may exert upon the mechanism of animal resistance as well as the spirocheticidal action of the agents employed.

CONCLUSIONS.

From the facts presented, it may be concluded that the existence of an infection with *Spirochæta pallida* does not constitute a bar in itself to the introduction and propagation of a second infection in the same animal; that, just as there is a period following a first inoculation during which a second infection may be implanted with the production of characteristic primary lesions, conditions may again arise in animals which have once become refractory to a second inoculation, that will favor the introduction of a new infection with the formation of lesions presenting the characteristics of an original or first infection.

Experimentally, such a state may be induced in rabbits with early but well marked primary lesions of the testicles by treatment with either arsphenamine or neoarsphenamine, hence, treated but uncured animals may be rendered as susceptible to a second cutaneous inoculation as a normal animal, and the manifestations of disease resulting from the second infection may be indistinguishable from those of a first infection.

SUMMARY.

Experiments were carried out on rabbits for the purpose of determining the effects of subcurative doses of arsphenamine and of neoarsphenamine upon the resistance of infected animals to reinoculation with *Treponema pallidum* and hence the possibilities of the occurrence of a second infection in treated but uncured cases of infection.

All the animals used were inoculated with the same virus, and the experimental tests were carried out when the first cycle of testicular reaction was nearing its height. The animals with the most marked testicular lesions were used for the basic experiment of treatment and reinoculation. The results of this experiment were controlled from four different standpoints: (1) the effect of the treatment employed upon the existing infection; (2) the immunity present at the time of treatment; (3) the virulence of the organisms used for reinoculation as compared with those causing the existing infection; (4) the comparative susceptibility of normal animals to the virus used for reinoculation.

The results obtained showed (1) that the treatment employed was insufficient to cure any of the therapeutic controls; (2) that the infected controls were highly refractory to a second inoculation; (3) that the treated animals were highly susceptible to a second inoculation and although not cured of their original infection, reacted to the second inoculation with the formation of lesions indistinguishable from those of a first infection; (4) that in certain instances the treatment given had rendered infected animals more susceptible to infection than the normal controls.

BIBLIOGRAPHY.

1. Jacobi, L., *Arch. Dermatol. and Syphilol.*, 1920, ii, 493.
2. Finger, E., and Landsteiner, K., *Arch. Dermatol. u. Syph.*, 1905, lxxviii, 335; 1906, lxxxi, 147; *Verhandl. deutsch. dermatol. Ges.*, 1907, ix, 251.

EXPLANATION OF PLATES.

The illustrations are reproductions of photographs which represent the objects at their natural size.

PLATE 73.

FIGS. 1 and 2. Reinoculation lesions of control animals.

FIG. 1. A lenticular papule at the base of the ear 15 days after reinoculation. The lesion did not progress beyond the point shown.

FIG. 2. 30 days after reinoculation. There is an indurated papule in the same location. These were the most marked lesions produced by reinoculation of the infected controls.

FIGS. 3 to 7. Reinoculation lesions following treatment with arsphenamine.

FIGS. 3 and 4. Characteristic chancres on the ear and sheath of a treated animal as they appeared 42 and 37 days after reinoculation.

FIGS. 5 and 6. Ear and sheath lesions of an animal with clinical relapse of the original lesions—37 days after reinoculation. The right testicle (Fig. 6) is diffusely enlarged and indurated and contains two well defined nodules; the left testicle is still atrophic but there is a small area of infiltration in the scrotum.

FIG. 7. The genital lesions of the same animal 12 days later. Note the marked increase of all lesions and the extensive necrosis of the lesion on the sheath.

PLATE 74.

FIGS. 8 to 12. Reinoculation lesions following treatment with neoarsphenamine.

FIGS. 8 and 9. These figures are from the same animal and represent conditions as they existed 42 days after reinoculation. The two lesions are about equally developed and show marked necrosis.

FIGS. 10 to 12. From a second animal. Figs. 10 and 11 are 42 days, and Fig. 12, 49 days after reinoculation.
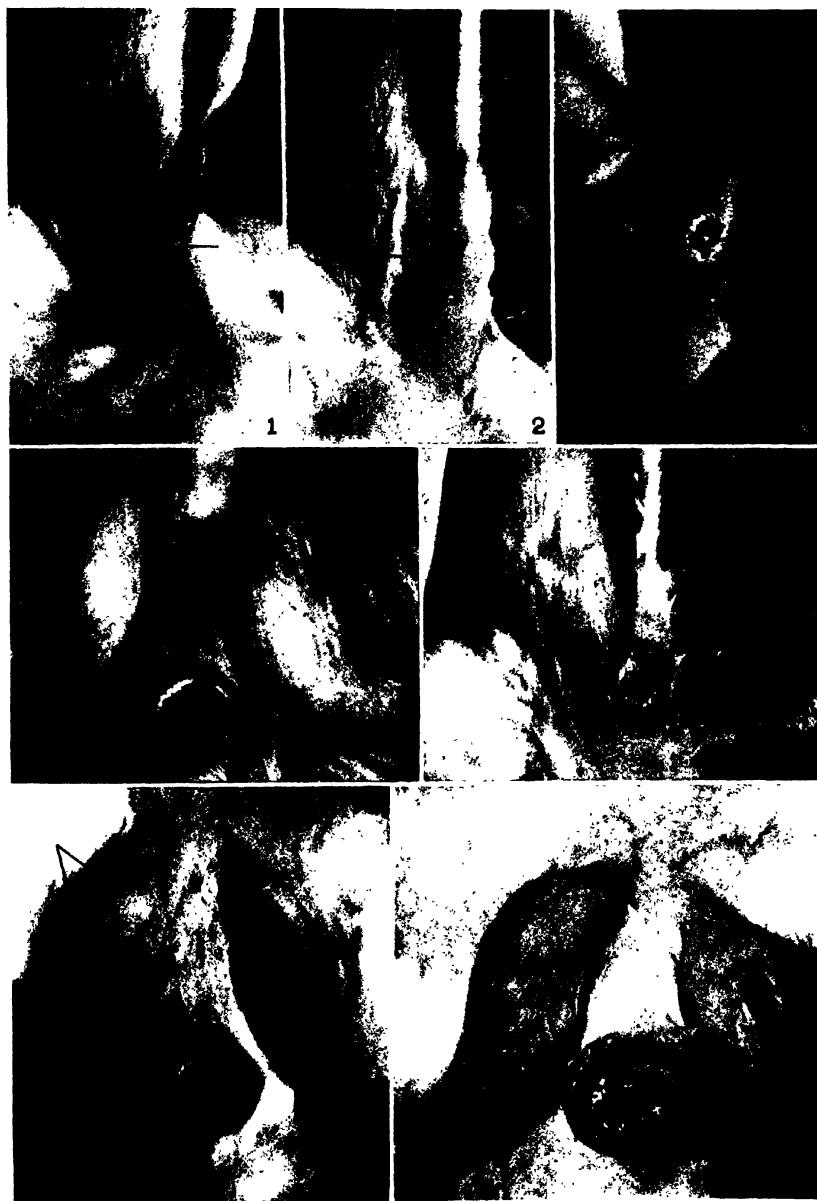
10

ETIOLOGY OF YELLOW FEVER.

XIII. Behavior of the Heart in the Experimental Infection of Guinea Pigs and Monkeys with Leptospira Icteroides and Leptospira Icterohæmorrhagiæ.

By ALFRED E. COHN, M.D., and HIDEYO NOGUCHI, M.D.

(*From the Hospital and Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 85 and 86.

(Received for publication, March 9, 1921.)

It is well known that in patients suffering from yellow fever the rate of the heart has a tendency to be slow. This phenomenon was regarded by Faget,[1] who was among the first to call attention to it, and by Touatre[2] as pathognomonic in this disease, especially in its early stage. In the second stage, the rate falls still further. It may be as low as 30 or 40 per minute, and may continue slow during convalescence. In fatal cases in the final stage it may be either rapid or slow. In the curves of patients published by Noguchi[3] and by Elliott,[4] these relations are 'seen. Sarti[5] gives, however, high rates for ten of the eleven cases he reports. It is said that death is seldom due to heart failure. At autopsy, lesions of the heart are not constantly found, except perhaps the degenerative changes and frequent

---

[1] Faget, J. C., Monographie sur le type et la spécificité de la fièvre jaune établie avec l'aide de la montre et du thermomètre, Paris, 1875.

[2] Touatre, J., Yellow fever; clinical notes, translated by Chassaignac, C., New Orleans, 1898.

[3] Noguchi, H., Etiology of yellow fever. I. Symptomatology and pathological findings of the yellow fever prevalent in Guayaquil, *J. Exp. Med.*, 1919, xxix, 547.

[4] Elliott, C. A., A clinical study of yellow fever. Observations made in Guayaquil, Ecuador, in 1918, *Arch. Int. Med.*, 1920, xxv, 174.

[5] Sarti, J., Contribucion al estudio de la fiebre amarilla, Tesis, Guatemala, 1919.

endocardiac or pericardiac ecchymoses associated with fever.   Recent studies on infectious jaundice[6] indicate, however, that in that disease also the pulse rate is usually slow in proportion to the temperature; one case is reported in which the pulse fell as low as 38.   This holds true even in cases without jaundice.
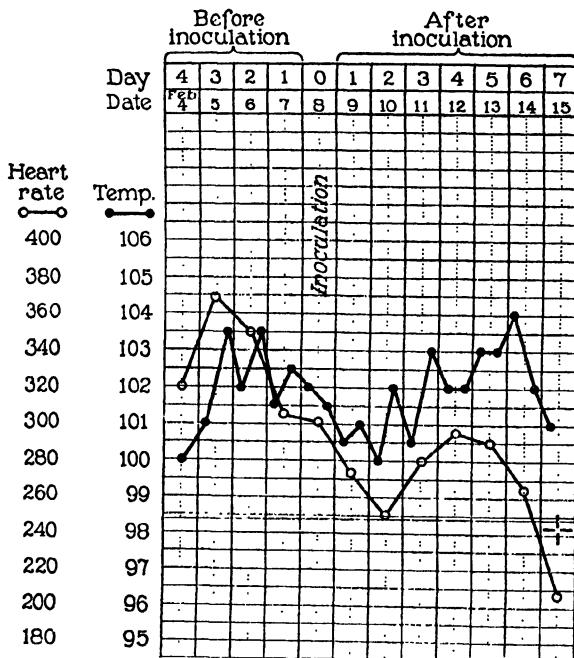
TEXT-FIG. 1. Temperature and heart rate curves of Guinea Pig 3, inoculated with *Leptospira icteroides*.

Since the successful transmission of yellow fever to animals, observations have been made of the rate and of the behavior of the heart in animals experimentally infected with *Leptospira icteroides* from yellow fever cases.   Records were taken electrocardiographically.

[6] Stokes, A., Ryle, J. A., and Tytler, W. H., Weil's disease (spirochætosis ictero-hæmorrhagica) in the British Army in Flanders, *Lancet*, 1917, i, 142. Ryle, J. A., Spirochætosis icterohæmorrhagica (formerly known as Weil's disease). A clinical analysis of fifty-five cases, *Quart. J. Med.*, 1921, xiv, 139.

By this method the rate can be accurately counted and the mechanism of slowing, if it occurs, can be analyzed. For comparison with the animals inoculated with *Leptospira icteroides* (yellow fever) a certain number of others infected with *Leptospira icterohæmorrhagiæ* (infectious jaundice) were included in the study.

Electrocardiograms were taken either for 1, 3, or 4 days before the day of inoculation. Exception to this procedure occurred in Guinea
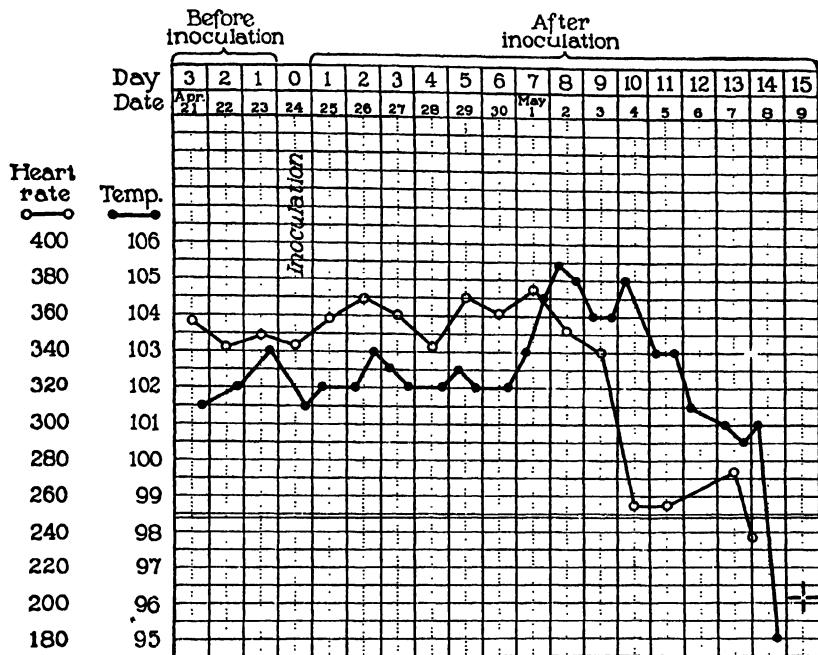
TEXT-FIG. 2. Temperature and heart rate curves of Guinea Pig 12, inoculated with *Leptospira icterohæmorrhagiæ*.

Pigs 4 and 6 and Monkey 3. The cardiac rate before inoculation of the guinea pigs was below 300 per minute three times in Guinea Pig 1, twice in Guinea Pig 2, and once in Guinea Pig 10. In the other nine guinea pigs it was always above 300, the range being from 302 to 371. In the monkeys the rate was above 400, except once in Monkey 2, and once in Monkey 5. The rate otherwise was above 400, the range being from 401 to 442.

In eleven animals there occurred a fairly consistent fall in rate either immediately after inoculation (Guinea Pigs 3, 7, and 9 and Monkey 2) (Text-fig. 1) or after a delay (Guinea Pigs 2, 4, 5, 8, and 12 and Monkeys 3 and 4) (Text-fig. 2). In several instances (Guinea Pigs 2, 4, 7, and 9) a rise occurred in the course of the disease. In each instance, however, there was a striking fall on the day of death, or during the days immediately preceding death. Guinea Pig 2
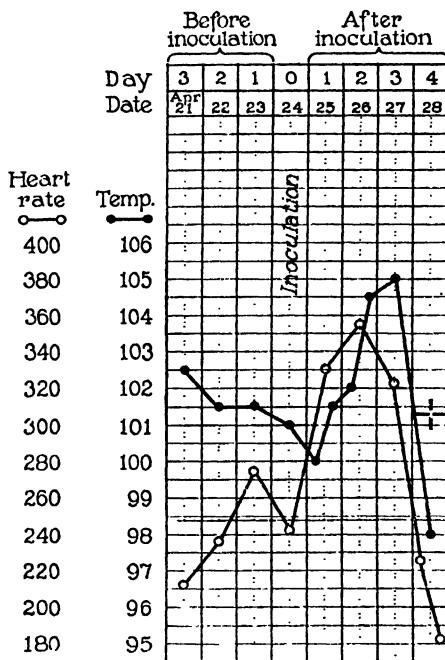
TEXT-FIG. 3. Temperature and heart rate curves of Guinea Pig 1, inoculated with *Leptospira icteroides* but with negative results.

appears to be an exception, but this is one of the animals in which low initial rates were recorded. The animals inoculated with *Leptospira icteroides* reacted, then, with a fall in rate. Those inoculated with *Leptospira icterohæmorrhagiæ* behaved in a similar manner (Table I).

In Guinea Pigs 1 (Text-fig. 3) and 6 the rates before inoculation were low, rose abruptly to high levels after inoculation, and then

fell. Guinea Pig 1 was inoculated with *Leptospira icteroides*, but died of a complicating peritonitis. Guinea Pig 6 ran an atypical course and died perhaps of a secondary infection.

This account leaves for consideration four animals, Monkeys 1 and 5 and Guinea Pigs 10 (Text-fig. 4) and 11. The monkeys did not develop disease of a nature that could be regarded as characteristic and are therefore offered as controls. They showed no striking
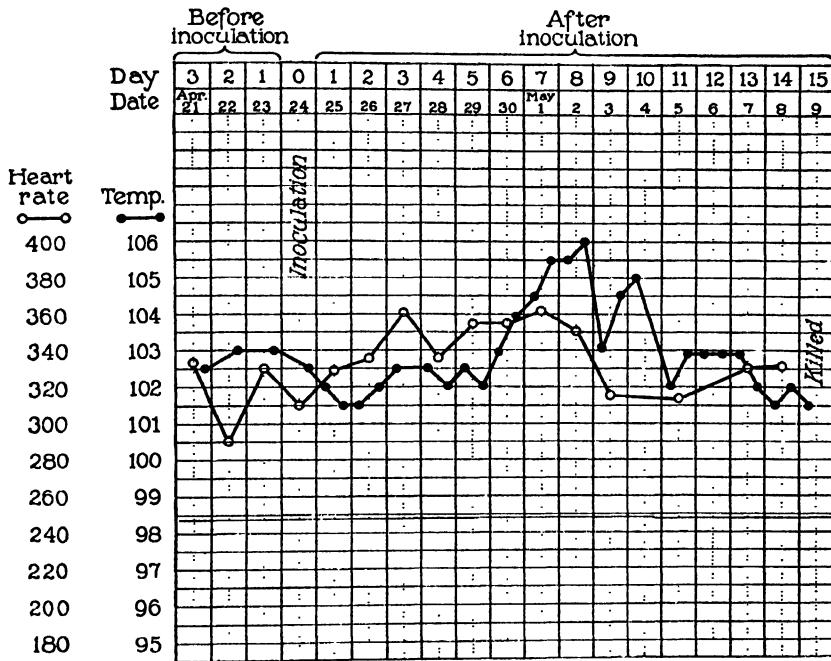
TEXT-FIG. 4. Temperature and heart rate curves of Guinea Pig 10, inoculated with *Leptospira icterohæmorrhagiæ* but with negative results.

change. in rate. Like observations are afforded by Guinea Pigs 10 and 11. They were killed after 14 days. The course of the disease in them was mild and the lesions were atypical. They are, therefore, also to be regarded as controls.

Mechanism of the Heart Beat.—During the control period preceding inoculation in the *icterohæmorrhagiæ* group, a single animal, Guinea Pig 12, showed an irregularity of the cardiac mechanism on one

TABLE I.

Records of the Pulse Rate of Animals Inoculated with Leptospira icteroides and Leptospira icterohemorrhagiae.

Animal No.	Days before inoculation.				Average.	Day of inoculation.	Days after inoculation.															Fall in rate.*	Irregularity.	Result.
	4	3	2	1			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15			
G. P.† 1	211	235	275	240	243	330	355	322	181	D.‡												−59	−	Died in 4 days. Negative: *L. icteroides* G§ 5. Peritonitis. (Text-fig. 3.)
" 2	221	256	335	271	292	340	331	350	278	286	315		D.									+44	−	Died in 7 days. Positive: *L. icteroides* G§ 5. Fever 3rd, 4th, 5th, and 6th days; peritonitis.
" 3	320	369	350	305	338	300	273	250	280	295	290	265	208	D.								−130	+	Died in 7 days. Typical: *L. icteroides* G§ 5. Fever 4th, 5th, and 6th days (Text-fig. 1).
" 4						266	275	303	330		265	233	D.									−33	+	Died in 6 days. Typical: *L. icteroides* G§ 6. Fever 3rd, 4th, 5th, and 6th days.
" 5	345	371	361	345	355	336	318	319	340	327	322	301	320	298	323	292	304					−51	−	Recovered in 10 days. Mild: *L. icteroides* G§ 6. Fever 5th day; mild.
" 6						211	458	375	311	257	D.											+46	−	Died in 4 days. Atypical: *L. icteroides* G§ 6. Fever 4th day; perhaps secondary infection.

																	Difference*		Remarks
G.P.	7	340	362	308	302	328	282	252	265	323	257	211	238	D.		−90	+	Died in 7½ days. Typical: *L. icteroides* G§ 6. Fever 3rd, 5th, and 6th days.
M.	1	405			375	385	400	369	363	376	324			"		−81	−	Died in 8 days. Negative: *L. icteroides* G§ 5. No fever; no typical lesion.
"	2	442	430	370	414	408	381	380	357	345	231	124		D.		−290	+	Died in 6 days. Typical: *L. icteroides* G§ 5. Fever 4th and 5th days.
"	3				446	338	442	440	440	385	333	137		D.		−309	+	Died in 7 days. Typical: *L. icteroides* G§ 6. Fever 3rd and 4th days.
G.P.	8	315	344	346	321	331	352	319	315	345	326	306	276	D.		−55	−	Died in 6 days. Typical: *L. icterohæmorrhagia* (Japanese). Fever 3rd, 4th, and 5th days.
" "	9	341	362	338	328	342	305	286	254	233	317	306	244	"		−98	−	Died in 6½ days. Typical: *L. icterohæmorrhagia* (Japanese). Fever 4th and 5th days.
M.	4	401			378	375	373	339	316	302	250			"		−151	−	Died in 7 days. Atypical(?): *L. icterohæmorrhagia* (Japanese). Moderate hemorrhage; no fever; no jaundice.

* Difference between the average of the control period and the final record.

† G. P. indicates guinea pig; M., monkey.

‡ D. denotes day of death.

§ *Leptospira icteroides* Guayaquil.

111

TABLE 1—*Concluded.*

Animal No.	Days before inoculation				Average	Day of inoculation	Days after inoculation															Fall in rate.	Irregularity.	Result.
	4	3	2	1			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15			
G. P. 10	333	289	332	318	309	327	334	363	335	357	355	362	352		313		314		330	331		+13	−	Killed in 14 days. Probably negative or very mild: *L. icterohæmorrhagiæ* (French). Fever 7th to 10th days; no jaundice; no hemorrhage; no lesions (Text-fig. 4).
" " 11	360	340	360	353	361	360	376	363	350	371	367	362	342		306		320		327	328		−25	−	Killed in 14 days. Mild: *L. icterohæmorrhagiæ* (French). Fever 7th to 14th days; intercurrent infection; no jaundice; no hemorrhage; no lesions.
" " 12	358	343	347	349	345	360	370	363	344	370	363	374	356		340		254		276	238	D.	−111	+	Died in 14 days. Atypical: *L. icterohæmorrhagiæ* (French). Fever 7th to 10th days; incubation 6½ days (Text-fig. 2).
M. 5	436	436	372	415	432	415	431	392	370	D.												−45	−	Died in 4 days. Negative: *L. icterohæmorrhagiæ* (French). Doubtful; irregular fever; slight hemorrhage in lungs; no jaundice.

occasion. During the 14 days following, with two exceptions, curves were taken daily. A recurrence of the arrythmia was not seen. During the course of the disease nothing remarkable in the behavior of the heart was recorded, although curves of the animals in this group which died were taken on the day of death (Guinea Pig 12 and Monkey 5) and on the day preceding (Monkey 4).

Of the yellow fever group, no irregularity was seen in any animal in the period preceding inoculation. A single guinea pig, No. 7, on the 2nd day afterward, showed an arrythmia (premature ventricular contractions). It died 5 days later but the irregularity did not recur. In four other animals, irregularities took place (Guinea Pigs 3 and 4 and Monkeys 2 and 3), but in two of them (Guinea Pigs 3 and 4) they occurred on the day of death, indeed during the terminal period. At this period Guinea Pig 3 showed heart block (Fig. 1). Monkey 2 (Fig. 2) began to show changes in the electrocardiogram on the day before death. The alterations are not different from those which have been described in connection with the death of the heart in other diseases. There was slowing of the auricles and the whole heart, and increase in the length of the auriculoventricular interval in Guinea Pig 3 and Monkey 2 (Fig. 2) and lengthened conduction time leading to block in Guinea Pig 3. In only two of the animals, Monkeys 2 and 3 (Fig. 3), did changes occur before the day of death. In both the change was detectable 2 days before death. Both showed slowing of the rate and alteration in the form of the ventricular complex in the electrocardiogram. An S wave not previously present appeared, and the T wave increased in size (Fig. 2).

It may be said, then, that the mechanism of slowing in all cases until the day of death was of a simple variety—a slowing of the whole heart, depending on the sinus, the pacemaker of the heart. On the day of death, although increased length of the auriculoventricular interval occurred, it led to heart block only once. Changes in the form of the electrocardiogram were seen twice (Monkeys 2 and 3, Fig. 3) in the course of the disease 2 days before death. In curves that were obtained during death of the heart changes were seen which resemble those usually found at this time.

CONCLUSIONS.

1. Slowing of the heart occurred in monkeys and guinea pigs during the febrile period of the experimental infection due to *Leptospira icteroides*. A similar reaction took place in animals inoculated with *Leptospira icterohæmorrhagiæ*.

2. The mechanism of slowing was usually due to slowing of the whole heart.

3. Once incomplete heart block was seen. Changes in the ventricular complex occurred four times.

EXPLANATION OF PLATES.

In the curves divisions of the abscissæ equal 0.04 second, divisions of the ordinates 0.1 millivolt. The electrodes were placed on the right fore and the left hind legs.

PLATE 85.

FIG. 1, *a* and *b*. Guinea Pig 3. (*a*) February 4, 1919. Control curve. (*b*) February 15. Auriculoventricular dissociation and a marked change in the ventricular complex are seen.

FIG. 2, *a* and *b*. Monkey 2. (*a*) April 21, 1919. Control curve. (*b*) April 30. Slowing, increase in height of R, S, and T waves, and increased auriculoventricular interval are seen.

PLATE 86.

FIG. 3, *a* to *g*. Monkey 3. (*a*) April 7, 1919. Control curve. (*b*) April 13. Day before death. S wave is increased. (*c*) April 14. Day of death. The R, S, and T waves are increased. The auriculoventricular interval is lengthened. (*d, e, f, g*) show increasing alteration in both auricular and ventricular parts of the curve. The ventricular alterations resemble those seen in dying hearts.

1a

1b

2a

2b
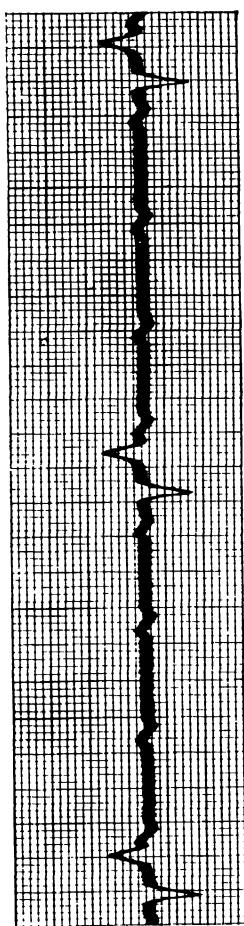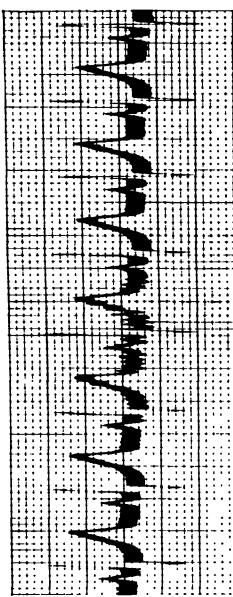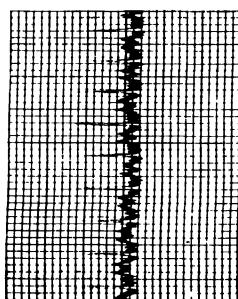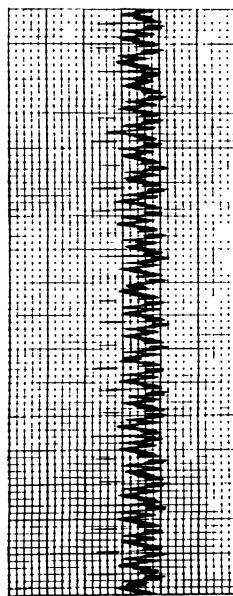
(Cohn and Noguchi: Etiology of yellow fever. XIII.)
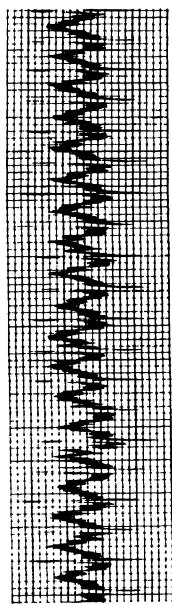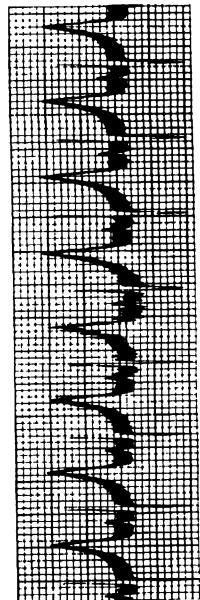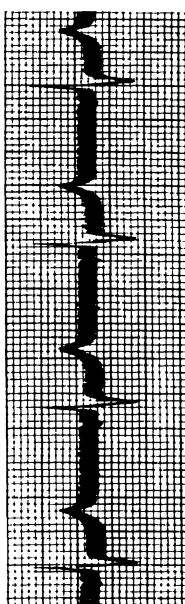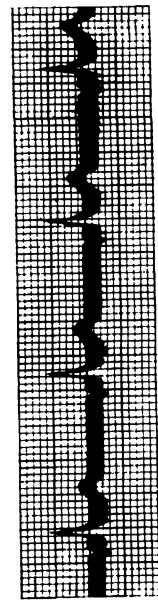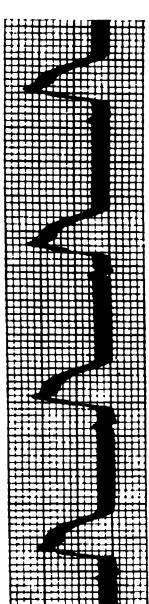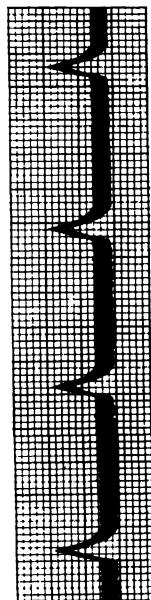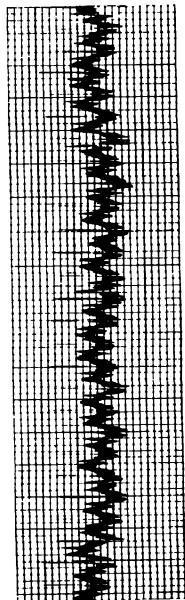
EXPERIMENTAL STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

IV. ANAEROBIC CULTIVATION.

BY PETER K. OLITSKY, M.D., AND FREDERICK L. GATES, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 95 TO 99.

In a series of reports in this Journal[1, 2, 3] and in *The Journal of the American Medical Association*,[4, 5] we have described the changes in the blood and lungs of rabbits and guinea pigs which follow the intratracheal injection of unfiltered and filtered nasopharyngeal secretions, obtained within 36 hours after onset, from patients ill with uncomplicated epidemic influenza. The activity of the injected material was traced to the presence of a substance possessing characters which could only be attributed to a living agent, not, however, of the nature of ordinary bacteria. In the earlier reports[1, 4] we referred to experiments on the cultivation of this living agent by anaerobic methods, and recently[5] we described the nature of the characteristic, visible bodies usually found by cultural methods to be present in the nasopharyngeal secretions during the early hours of epidemic influenza in man and in the lung tissue of affected animals. The present paper describes these cultivation experiments in greater detail.

[1] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 125.
[2] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 361.
[3] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 373.
[4] Olitsky, P. K., and Gates, F. L., *J. Am. Med. Assn.*, 1920, lxxiv, 1497.
[5] Olitsky, P. K., and Gates, F. L., *J. Am. Med. Assn.*, 1921, lxxvi, 640.

Sources of Material.

The characteristic bodies were first observed in November, 1918, in strictly anaerobic cultures of the filtered nasopharyngeal secretions of an influenza patient in the early hours of the disease. Since that date successful cultivation experiments have been carried out with material ultimately derived from all of the seven cases of influenza from which active material was transmitted to animals. Numerous primary cultures, directly from human sources or from the lung tissues of affected animals, have been followed by hundreds of sub-plants, until at present certain strains are extant in the eighteenth generation.

The materials subjected to cultivation, essentially the same as those injected in the transmission experiments, were obtained from a variety of sources. During the two epidemic waves of influenza, that of 1918–19 and that of 1920, nasopharyngeal secretions were collected from patients with the uncomplicated disease, both in the early hours of the affection and in the later stages, including convalescence. During the first epidemic filtered and unfiltered material was also obtained from the lungs, at autopsy, of patients who had succumbed to secondary or concurrent pneumonias. In the course of the various series of transmission experiments with rabbits and guinea pigs, the lungs of affected animals were cultured both aerobically and anaerobically as a routine. Usually this material was not filtered. Sometimes the tissues were ground in saline solution and filtered in order to remove bacteria of the ordinary species. As a routine also, portions of the lungs of affected animals were preserved in sterile 50 per cent glycerol. These glycerolated specimens, after preservation for periods up to 14 months, were directly cultured or injected intratracheally into rabbits, from whose lungs cultures were made at autopsy.

The control materials for cultivation experiments were likewise similar to those used in control transmission experiments in animals. They consisted of unfiltered and filtered nasopharyngeal secretions from healthy persons during the two epidemic waves, the interepidemic recession, and the postepidemic period. Nasopharyngeal washings from persons suffering from acute coryza were also cultured

during the non-epidemic intervals. The normal lungs of uninocu-
lated rabbits, the diseased lungs of stock rabbits who fell ill of other
infections, the lungs of rabbits injected with control materials, and,
finally, each of the various ingredients of the culture medium were
subjected to anaerobic cultivation as controls.

Methods of Cultivation.

All the methods of anaerobic cultivation employed involved the
use of fresh sterile kidney tissue in tubes or flasks of human ascitic
fluid or ascitic fluid agar, usually under a vaseline seal.

In the early cultivation tests sterile paraffin oil was used as a seal
instead of vaseline, and the tubes were incubated in an anaerobic jar
set up as described by McIntosh and Fildes.[6] The decolorization of a
tube of methylene blue in broth, included in the jar, indicated the
establishment of anaerobic conditions. In later experiments the
complete exclusion of oxygen by a vaseline seal permitted the rapid
production and the maintenance of anaerobic conditions by the
reducing substances in the medium.[7] The use of the anaerobic jar
was, therefore, discontinued.

For primary isolations and routine cultivations the culture tubes
were set up as follows: Relatively large pieces (0.6 to 0.8 gm.) of
sterile normal rabbit kidney tissue were placed in test-tubes measur-
ing 20 by 1.5 cm., one piece to each tube. Then 1 cc. of suspected
fluid or 0.5 cm. of affected lung tissue was placed directly on the
kidney tissue. These materials were covered with 8 to 10 cc. of
sterile human ascitic fluid of a hydrogen ion concentration of 7.8 to
8. Ascitic fluids of a higher alkalinity were discarded. Sealed with
2 cc. of melted sterile vaseline and stoppered with cotton, the tubes
were incubated at 37°C. for 8 to 12 days.

For special purposes this routine culture method was modified in
two particulars. Occasionally tubes of a semisolid medium or mass
cultures in flasks were employed, especially for subplants. The semi-
solid medium consisted of 1 part of beef infusion (2 per cent) agar,
pH 7.4, to 2 parts of human ascitic fluid, mixed in a flask at 40°C.

---

[6] McIntosh, J., and Fildes, P., *Lancet*, 1916, i, 768.
[7] Gates, F. L., and Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiii, 51.

and pipetted onto the kidney tissue and inoculum in the tall test-tubes. The medium for mass cultures was slightly modified from that described by Flexner, Noguchi, and Amoss[8] for the cultivation of the globoid bodies of poliomyelitis. In each Florence flask, of 50 cc. capacity, was placed one-fourth of a moderate sized rabbit kidney. The sections were cut across the entire kidney and placed with the cut surface parallel to the base of the flask. After inoculation the kidney was just covered (10 cc.) with the semisolid ascitic fluid medium described above and the agar was hardened by immersion of the flask for 15 minutes in cold water. Then the flask was filled to the neck with a mixture of 1 part of beef infusion broth, pH 7.4, and 2 parts of ascitic fluid. A seal of vaseline, 1 cm. deep, and a cotton stopper completed the preparation.

An advantage of the vaseline seal over the anaerobic jar was found in the ease with which specimens for examination or transplant could be obtained with a capillary pipette without exposure of the medium to the air. After puncture the seal was restored by gentle heat—just sufficient to melt a portion of the superposed vaseline.

All the earlier inoculations were made directly on the kidney tissue before the medium was added. More recently the incubation period has been shortened and growth facilitated by inoculating sterile preparations set up a day or two in advance, to permit the establishment of anaerobic conditions under the vaseline seal. The inoculum is placed in the vicinity of the kidney by means of a capillary pipette.

In primary cultivations and early subplants the suspected material was distributed among three to six culture tubes. Frequently only a few tubes of several in an initial cultivation showed growth. But apparent failure in the initial cultivation might be followed by success in a subplant. Hence at least two negative cultivations in succession were required before a culture series was discarded as negative.

Appearance of Positive Cultures.

The first evidence of the multiplication of a living substance in primary cultures in fluid medium was observed in the presence of a faint haze, first visible about the 5th day, extending upward about

[8] Flexner, S., Noguchi, H., and Amoss, H. L., *J. Exp. Med.*, 1915, xxi, 91.

1 cm. from the level of the kidney. This faint even cloud gradually became denser, reaching its maximum about the 8th day, when it approximated 3 cm. in depth. At the same time the clear supernatant fluid developed a characteristic very faint opalescence, often hardly discernible. On standing at room temperature the cloud gradually settled down, in the course of 2 weeks, to the region of the kidney tissue, leaving a clear supernatant fluid.

Initial cultures in a semisolid medium usually failed to show signs of growth. Subplants from the fluid medium developed slowly in semisolid cultures with the formation of almost microscopic colonies, too small to be defined with exactness. The evidence of growth was an even clouding of the medium extending to 3 to 2 cm. from the surface.

In mass cultures of well established strains a turbidity appearing throughout the semisolid layer about the 3rd day was followed by a diffuse clouding of the fluid portion by the 5th day. On standing at room temperature the cloud gradually subsided to the vicinity of the agar, forming a dense homogeneous nebulous layer on its surface (Fig. 1).

Morphology and Staining Reactions.

The greatest concentration of cultivable bodies for microscopic examination was obtained from the very bottom of the culture tubes. 0.3 to 0.4 cc. of the cloudy sediment was drawn up in a capillary pipette. After removing the adherent vaseline from the pipette with gauze the first two drops of fluid were discarded and subsequent drops were spread evenly on a slide in thick films. The films were then dried in an incubator at 37°C., fixed with gentle heat—three rapid passages through the Bunsen flame—and stained by the chosen method.

Of all the stains tried, well ripened Loeffler's alkaline methylene blue proved the most satisfactory. The preparation was flooded with the dye and steamed very gently over a flame for 2 minutes. The slide was thoroughly washed with running tap water and allowed to dry in the air.

Prepared in this manner, the films from typical cultures revealed minute bodies of regular morphology, stained a deep purple, and

clearly differentiated from the background of pale blue protein precipitate. The bodies were often exceedingly numerous, and with careful focusing, they stood out in sharp relief. Usually solitary, they were often found in diplo forms, and occasionally in short chains of three or four members. Clumps occurred also, especially in older cultures, the discrete definitely stained bodies forming masses from a blood platelet to a leucocyte in size. Viewed with the highest powers of the microscope, the bodies were seen to be two to three times longer in one direction than in the other. They were, therefore, bacilloid rather than coccoid. Thus they were differentiated sharply from the globoid bodies of poliomyelitis, which they approached in size. Their long axis measured 0.15 to 0.3 microns. Occasionally longer individuals were seen, but the organisms showed little tendency to pleomorphism and were characterized by uniformity in size and shape. Irregular staining reactions have not been encountered. No granules, clubs, spores, or involution forms have been seen. The bodies in one culture in its eighteenth generation are morphologically identical with those in the initial specimen (Figs. 2 to 5).

Occasionally unmistakable clumps or colonies developed in the region of the kidney (Fig. 5), but when few in number the bodies were sometimes obscured by protein or stain precipitates (Fig. 4). Under such circumstances, the unaccustomed eye made them out with difficulty. For this reason stains which showed any tendency to precipitation on the slide were found unsuitable for their demonstration. Thus, although the bodies were died lavender with Giemsa's, Wright's, and Manson's stains, the preparations were as a rule unsatisfactory. Carbolthionine (Nicolle), carbolfuchsin, steaming safranine, and steaming fuchsin were likewise unsuitable. All the strains examined decolorized uniformly by Gram's method. The counterstain, safranine, required steaming. The Gram-negative reaction was a constant feature of young and old cultures.

The dark-field microscope has not afforded a satisfactory method of examination on account of the similarity in size of the cultivable organism to the familiar dancing bodies of control preparations.

Filterability.

The cultivable bodies, even in remote generations, have been found to pass Berkefeld V and N candles. They are, therefore, to be classed among the filter passers.

Cultural and Biological Characters.

The strict anaerobic and nutritive requirements of the cultivable bodies have necessarily limited the study of their cultural reactions. Certain conclusions have been drawn from repeated experiments.

Final Hydrogen Ion Concentration of Cultures.—The incubation of sterile kidney tissue in ascitic fluid has been shown to change the hydrogen ion concentration of anaerobic tissue cultures toward the acid side.[7] In our experiments the final hydrogen ion concentration of growing cultures and of uninoculated controls was the same; namely, pH 7.4 to 7.8. The organisms failed to grow in media of a pH of 8 or 7, which appear to be the outside limits of the favorable range.

Action on Carbohydrates and Alcohols.—Growth took place in the presence of dextrose, maltose, lactose, saccharose, inulin, and mannitol. No observable amounts of acid or gas were produced. The media containing dextrose and maltose (1 per cent) showed a heavier cloud than usual, but this may or may not have corresponded to a more active multiplication of the organisms. No characteristic odor was detectable in any of the cultures.

Symbiosis.—In the course of the experiments certain culture tubes, inoculated with unfiltered lung tissue, yielded growths of ordinary bacteria in addition to the characteristic bodies seen in the original culture tubes and later demonstrated in pure culture with filtrates of the mixed material. It thus appears that this organism can develop in symbiosis with *Bacillus pfeifferi*, the pneumococcus, *Streptococcus hæmolyticus*, *Streptococcus viridans*, and staphylococci. In a few experiments deliberate mixtures of these bacteria with the influenzal bodies were cultivated, and the organisms were subsequently separated by plating and by filtration.

Resistance.—No growth has been obtained in subplants of cultures heated to 56°C. for ½ hour or longer.   Exposure to chloroform vapor for 1 to 1½ hours apparently destroys the organism.   Viable organisms have been found, however, in fluid, semisolid, and mass cultures kept at room temperature, 20–24°C. (68–76°F.) for periods up to 6 months.

In these respects the resistance of the cultivated bodies is similar to that of the active agent in glycerolated specimens of the lung tissue from affected rabbits.[2]

Enumeration of Positive Cultures and the Sources from Which They Were Obtained.

In the earliest experiments, before a precise technique for culturing and demonstrating the cultivable bodies was developed, it may be presumed that some active materials gave negative results.   Hence the following enumeration of successful cultivations is of more significance from the positive than from the negative point of view, and cannot be regarded as an indication of the actual incidence of these cultivable bodies in epidemic influenza in man.   This is particularly true in view of the fact that cultural experiments were not accepted as positive unless at least two generations of the cultivable bodies were obtained.

Cultivation experiments were attempted with the filtered nasopharyngeal washings of eleven patients with uncomplicated epidemic influenza during the first 36 hours of the illness.   Of these cultivations, six gave positive results.   Five strains were obtained from eight patients in the 1918–19 epidemic and one strain from three patients in the recurrence of 1920.

Material from twenty-eight other patients was cultured during the later stages of the disease—from the 48th hour to convalescence. Only one culture yielded a growth.   This material was obtained 48 hours after onset from a patient who died 2 days later from a secondary pneumonia.

The filtered nasopharyngeal washings of four patients suffering from pneumonias secondary to epidemic influenza were apparently negative.   No growth was obtained with filtrates of the lung

tissue, at autopsy, of two patients who succumbed to the secondary pneumonias.

Although the cultivation of these peculiar anaerobic bodies from the lung tissues of a large number of affected rabbits and guinea pigs in the transmission experiments already described[1, 2, 3] is presumptive evidence that this organism was probably the causative agent in the lesions produced, it is nevertheless important to correlate the presence of these organisms in the human nasal washings with the pathogenicity of these washings for rabbits.

The transmission experiments were initiated with the nasopharyngeal washings of seven patients, all in the first 36 hours of illness. Three of these specimens, filtered and cultivated, yielded strains of the characteristic organism. The specimens from three other patients failed to produce a growth. The seventh specimen was not cultivated. An eighth specimen, obtained early, appeared to be negative both in cultivation and animal transmission experiments. Three strains of the cultivable bodies were obtained from specimens of nasopharyngeal washings which were not used for transmission experiments in rabbits.

Strains were ultimately derived, however, from all seven of the patients enumerated above by cultivation of the lung tissues of rabbits and guinea pigs affected in animal transmissions of the active nasopharyngeal material.

Beside the unfiltered and the filtered nasopharyngeal washings and the fresh lung tissues of affected rabbits and guinea pigs, a third source of active material, pathogenic for rabbits, was rabbit lung tissue which had been preserved in sterile 50 per cent glycerol for periods up to 9 months.[2]

None of the specimens of glycerolated lung which were directly cultivated yielded growths of the specific organism. We have already reported[2] the activity of certain specimens of the glycerolated material in initiating characteristic lesions when injected intratracheally in rabbits. From the fresh lung tissues of these affected animals, or their successors in the line of animal passage, the anaerobic bodies were cultivated in a number of instances. In this way the primary cultivation of rabbits' lungs from eleven series of experiments in which the active material had previously been immersed in glycerol

for 5 days to 9 months, yielded three cultures of these bodies; in one instance the length of glycerolation was 5 days, in another 1 month, and in the third 9 months.   The two original sources of active material used in the above eleven series were Case 16,[1] representing the first epidemic period, and Case 26,[1] representing the second.

Control Cultivation Experiments.

Control cultivation experiments were made directly with the unfiltered or filtered nasopharyngeal secretions of twenty patients free from influenza.   Eight of these patients were suffering from an acute coryza in the early or late stages.   The control materials were collected in the epidemic, interepidemic, and postepidemic periods.   None of these specimens yielded the cultivable bodies found in six of the eleven early cases of influenza examined.

Control cultures of the following materials also uniformly failed to yield growth of these bodies: the lung tissues of six stock rabbits which died of accidental or epidemic infections such as snuffles or pneumonia; uninoculated tubes of 36 samples of human ascitic fluid and portions of all the rabbit kidneys used in the culture media; and the lung tissues of 60 rabbits, either normal or injected intratracheally with control materials in the course of the transmission experiments. The control materials injected in these rabbits included normal rabbit lung tissue, saline solution, human ascitic fluid, rabbit serum, and ordinary bacteria.

Inoculation of Rabbits and Guinea Pigs with the Cultivable Bodies.

For a study of the effects of culture injections on animals, mass cultures[9] were generally used.   Because of pressure of other experi-

[9] Mass cultures were prepared for inoculation as follows: The vaseline seal was removed from the Florence flask with a sterile wire and the fluid part of the culture centrifuged for 20 minutes at 1,500 revolutions per minute or until the supernatant fluid was clear.   The fluid was then decanted, and the small button-like deposit of the growth left at the bottom of the tube was resuspended in saline solution.   The centrifugation was repeated, the clear, supernatant saline solution was removed, and the small amount of sediment was again suspended in 4 cc. of saline solution, of which 3 cc. were used for the experiment.

ments most of the animal inoculations had to be deferred so that they were finally done with well established cultures several generations removed from the original human source.

When this part of the work was undertaken there were available for study three separate strains of cultivable bodies, two from the first epidemic and one from the second. These three strains were represented by cultures derived from nine different sources. One, a human strain, was the seventeenth generation subplant of a culture of the filtered nasopharyngeal secretion of Case 17[1] of the first epidemic. The others were first to seventh generation cultures of the lungs of different rabbits which had been injected with glycerolated lung tissue from earlier animals in the transmission series. Four of these cultures were thus originally derived from Case 16 and three were from Case 17 of the first epidemic. One came originally from Case 26 of the second epidemic of 1920.

Of these nine cultures with which rabbit passages were again initiated, only one, derived from rabbit lung tissue, and then in the third generation, failed to produce the effects regarded as typical for the active material in the earlier transmission experiments. The results of the intratracheal injection of the growth of mass cultures, in doses of 3 cc. were so uniform and familiar that a common description will suffice for the entire series.

On examination 24 hours after inoculation, the rabbits showed a rise in temperature and usually a conjunctivitis, varying from simple injection of the palpebral conjunctiva to a marked injection of the palpebral and ocular conjunctivæ. These signs were accompanied by a definite and often marked leucopenia, the result of a depression of the mononuclear cells (Text-figs. 1 and 2). In the animals which were kept for observation, these conditions persisted for 2 to 3 days, when the animals returned to normal. When the rabbits were killed during the reaction, a characteristic pathological picture was revealed in the respiratory organs.

Only the respiratory organs were visibly affected. The lungs (Figs. 6 and 7) were voluminous with edema and emphysema. Numerous hemorrhages were to be seen on the surface, either diffuse or discrete, and often in the form of minute petechiæ. The pleuræ were not

involved.   On section of the lungs, the cut surface dripped a frothy blood-stained fluid, evidence of a hemorrhagic edema.   Hemorrhages similar to those which had reached the surface were scattered through the parenchyma.   The trachea and bronchi showed a muco-purulent exudate covering an exfoliated and hemorrhagic epithelium.

Microscopic sections (Figs. 8 and 9) confirmed the gross observations.   The hemorrhages, diffuse or discrete, were located in the interalveolar tissue, which was distended with edema and torn by emphysema.   The interalveolar structures were also infiltrated to some degree with a cellular exudate consisting of mononuclear cells and some polymorphonuclear cells with large eosinophilic granules. Large cells of the respiratory epithelial type, probably desquamated bronchial epithelium, and numerous erythrocytes were seen in the parenchyma.   No pneumonic consolidation was present.

The bronchi showed thickened, hyperemic walls and their lumina were partly filled with erythrocytes, leucocytes, and fragments of exfoliated and necrotic epithelium.   The lung capillaries were distended with blood.

As noted above, eight series of animal transmission experiments were initiated by the intratracheal injection in rabbits of pure cultures of the anaerobic bodies.   From the lung tissues of rabbits in seven of these series the anaerobic bodies were recovered in pure culture.

Both the strain obtained directly from the filtered nasal washings of Case 17, then in its seventeenth generation, and the strains derived from Cases 16, 17, and 26[1] after rabbit passage and glycerolation, produced the typical effects described above.   The following protocols illustrate the similarity of the clinical effects produced by the human and the rabbit strains.

Protocol 1.—Preliminary observations on a rabbit for 2 days prior to inoculation gave the following results: temperature 39° and 38.9°C.; total leucocytes 15,200 and 16,000; mononuclear cells 5,168 and 7,360.   Oct. 18, 1920.   Inoculated intratracheally with 3 cc. of the growth of a second generation mass culture from the fifth rabbit passage of material from Case 16.   Oct. 19.   Temperature 39.6°C.; total leucocytes 8,000, of which 2,640 were mononuclears.   Oct. 20. Temperature 39.6°C.; total leucocytes 10,400, of which 2,808 were mononuclears (Text-fig. 1).   Rabbit killed.   The lungs showed the hemorrhagic, edematous changes regarded as typical.

Another rabbit injected with the third generation mass culture of the same strain showed a similar picture of leucopenia and mononuclear depression. The rabbit was allowed to recover. This condition lasted for 3 days (Text-fig. 2).
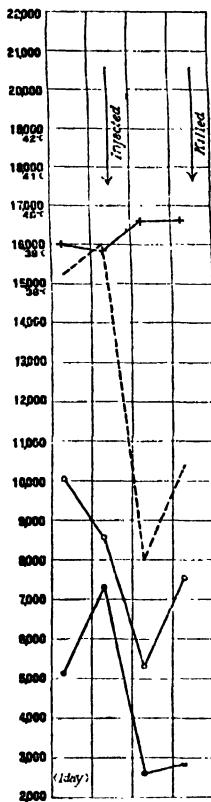
TEXT-FIG. 1.   TEXT FIG. 2.
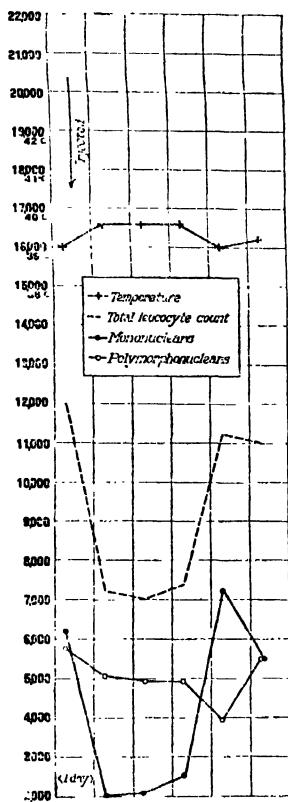
TEXT-FIG. 1. Effect on blood and temperature of the intratracheal injection of the cultivable bodies in a rabbit. The rise in temperature and the leucopenia due to mononuclear depression are noteworthy.

TEXT-FIG. 2. The same as Text-fig. 1 except that in this case the animal was allowed to recover. The persistence of the effects for 3 days is seen.

The next protocol is presented to show the effect of inoculation of a strain obtained by cultivating the nasopharyngeal secretions from a human case.

Protocol 2.—Jan. 6, 1921. A rabbit with normal temperature, 39°C., total leucocytes 10,400, of which 4,472 were mononuclears, was injected intratracheally with 3 cc. of the growth in the seventeenth generation mass culture of a strain from the filtered nasopharyngeal washings of Case 17.   24 hours later the animal showed a mild conjunctivitis, temperature 39.5°, total leucocytes 6,400, of which 1,152 were mononuclears. Jan. 8. The conjunctivitis was severe, total leucocytes 8,000, of which 2,400 were mononuclears. The lungs showed the lesions regarded as typical. The anaerobic bodies were recovered in pure culture from the lung tissue. The injection of this culture produced similar effects in a second rabbit, from the lung tissues of which the strain was again recovered.[10]

Several series of experiments were made with guinea pigs instead of rabbits. In this species the response to the intratracheal inoculation of cultures was similar to that obtained in rabbits, and from the affected lungs of the guinea pigs, the cultivable bodies were recovered.

Among the specimens of affected lung tissue preserved in 50 per cent glycerol were several which had come from rabbits injected with cultures of the anaerobic bodies. Although subsequent direct cultivations of these preserved specimens gave negative results, the same bodies were recovered from rabbit passages of the glycerolated material containing them. We thus have evidence that the bodies themselves do withstand glycerolation. The lungs from which these bodies were recovered by animal passage were immersed in glycerol for periods up to 4 months.

DISCUSSION.

These experiments seem definitely to connect the cultivable bodies with the clinical effects and lesions induced in rabbits and guinea pigs by the intratracheal injections of nasopharyngeal washings from patients with uncomplicated epidemic influenza.

From the lung tissues of such affected animals the morphologically and culturally characteristic bodies have been obtained in pure culture on special media by a strictly anaerobic technique. The bodies have been cultivated in successive generations without change in character. When injected intratracheally into rabbits and guinea

[10] The relation of ordinary bacteria in regard to their ability to produce concurrent or secondary infections in the presence of the cultivable bodies will form the basis of another paper.

pigs, they have given rise to pathological lesions in all respects similar to those from which they were obtained.

From the lesions the typical bodies have again been recovered in pure culture by the method employed for their primary isolation. Comparison of the strains thus derived from animal passages with those obtained directly from filtered human nasopharyngeal washings shows them to be identical in morphology and cultural characters. Finally, both the active material of the transmission experiments and the cultivable bodies obtained from similar sources withstand glycerolation and pass through Berkefeld V and N filters.

We feel, therefore, that the active material, pathogenic for rabbits and guinea pigs, found in the nasopharyngeal secretions of patients in the early hours of uncomplicated epidemic influenza has been identified in the anaerobic organism described in this paper.

It would, of course, be a simple matter to announce the inciting or etiological agent of epidemic influenza in man to be the minute, bacilloid organism here described. At present such a course does not seem desirable even though the clinical and pathological effects induced in the rabbit simulate so closely the phenomena found in epidemic influenza in man. Apparently we are at the threshold of our knowledge of a group or class of minute microorganisms which the anaerobic Smith-Noguchi technique has thrown open to exploitation. It seems wiser, therefore, to defer decision of the precise relation which the species described in this and previous communications bears to epidemic influenza until further experience is obtained.

In the meantime it is desirable to give the microorganism a name, and since a striking feature of its effect in rabbits is to diminish the resistance of the lungs to the action of ordinary pathogenic bacteria, as will be shown in a forthcoming paper, the name of *Bacterium pneumosintes* is proposed (from πνεύμων, lung, + σίντης, injurer, or devastator).

SUMMARY AND CONCLUSIONS.

From the filtered nasopharyngeal washings of patients in the first 36 hours of uncomplicated epidemic influenza and rarely in later stages of the disease, we have cultivated a minute bacilloid body,

Bacterium pneumosintes, 0.15 to 0.3 microns in length, of constant cultural characters and capable of indefinite propagation on artificial media. This, organism, not of the nature of ordinary bacteria, was also recovered in pure culture from the unfiltered and filtered lung tissue of rabbits and guinea pigs inoculated with unfiltered and filtered nasopharyngeal washings of early influenza cases, both from the first epidemic of 1918–19 and from the second one of 1920. The organism grows only under strictly anaerobic conditions, passes Berkefeld V and N filters, and withstands the action of sterile 50 per cent glycerol for a period of months.

It has been recovered from cultures contaminated with a variety of ordinary bacteria such as *Bacillus pfeifferi*, pneumococci, streptococci, and staphylococci, and has been experimentally cultivated in symbiosis with them.

Similar cultivation of control materials uniformly failed to yield growths of this organism. The materials tested consisted of the unfiltered and filtered nasopharyngeal washings of persons free from influenza, some of whom were suffering from acute coryza, the lung tissue of normal rabbits and of rabbits with bacterial respiratory infections, and the uninoculated media.

The intratracheal injection in rabbits and guinea pigs of mass cultures of this organism has induced effects on the blood and lungs of these animals which are not to be distinguished from those obtained with the nasopharyngeal secretions of patients in the early hours of epidemic influenza. From the pulmonary lesions thus induced the same organism has been recovered in pure culture, and has been found to cause similar lesions on subsequent animal passage. Its pathogenicity is not lost by prolonged artificial cultivation.

Our experiments indicate that the cultivable bodies obtained directly from human nasopharyngeal washings and from affected rabbit lungs are strains of the same organism. This organism appears to be the source of the reactions which occur in experimental animals —rabbits and guinea pigs—as a result of the intratracheal injection of nasopharyngeal washings obtained during the early hours of uncomplicated epidemic influenza in man.

EXPLANATION OF PLATES

PLATE 95.

FIG. 1. The construction of mass culture medium, to the left, and the growth of the cultivable bodies therein, to the right. The growth is derived from a rabbit's lungs after 8 days incubation. The turbidity of the semisolid and the haziness of the fluid layers are noteworthy. Actual size.

PLATE 96.

FIG. 2. The cultivable bodies in the eighth generation. Culture obtained from a rabbit's lung into which was injected material, after 9 months immersion in glycerol, originally derived from the filtered nasopharyngeal secretions from a case of epidemic influenza. Stained with ripened Loeffler's alkaline methylene blue. × 1,000.

FIG. 3. The bodies in Fig. 2 highly magnified. Stained similarly. × 3,000.

FIG. 4. Comparative size of the cultivable bodies. An aerobic streptococcus and a chain of poliomyelitis globoid bodies, indicated by an arrow, have been superimposed. The cultivable bodies are very minute, uniform, and lightly stained, and are to be differentiated from the small irregular masses, deeply stained, which are protein precipitates. All × 1,000.

FIG. 5. The cultivable bodies in colony formation. × 1,000.

PLATE 97.

FIG. 6. The gross lesions of the lungs of a rabbit inoculated intratracheally with the first generation of the cultivable bodies. The hemorrhages, edema, emphysema, and absence of pneumonic consolidation are noteworthy. Natural size.

FIG. 7. These lungs were obtained from a rabbit which was inoculated with the lungs pictured in Fig. 6. The more intense lesions of this second passage of the cultivable bodies are seen, especially the hemorrhagic edema of the right lung. Natural size.

PLATE 98.

FIG. 8. Microscopic section of the lung lesions in a rabbit, caused by injecting the cultivable bodies intratracheally. The edema, the emphysema, the hemorrhages, and the cellular exudate are noteworthy. × 240.

PLATE 99.

FIG. 9. Another field of the same section shown in Fig. 8, demonstrating particularly the bronchial lesions. The necrotic and exfoliated bronchial epithelium and the edema of its walls are to be seen. The general edema and the vessel filled with blood may be observed. × 240.

FIG. 1.
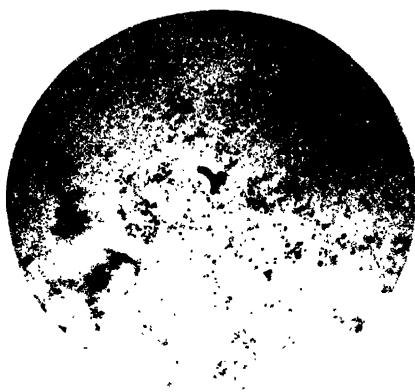
Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.

Fig. 7.
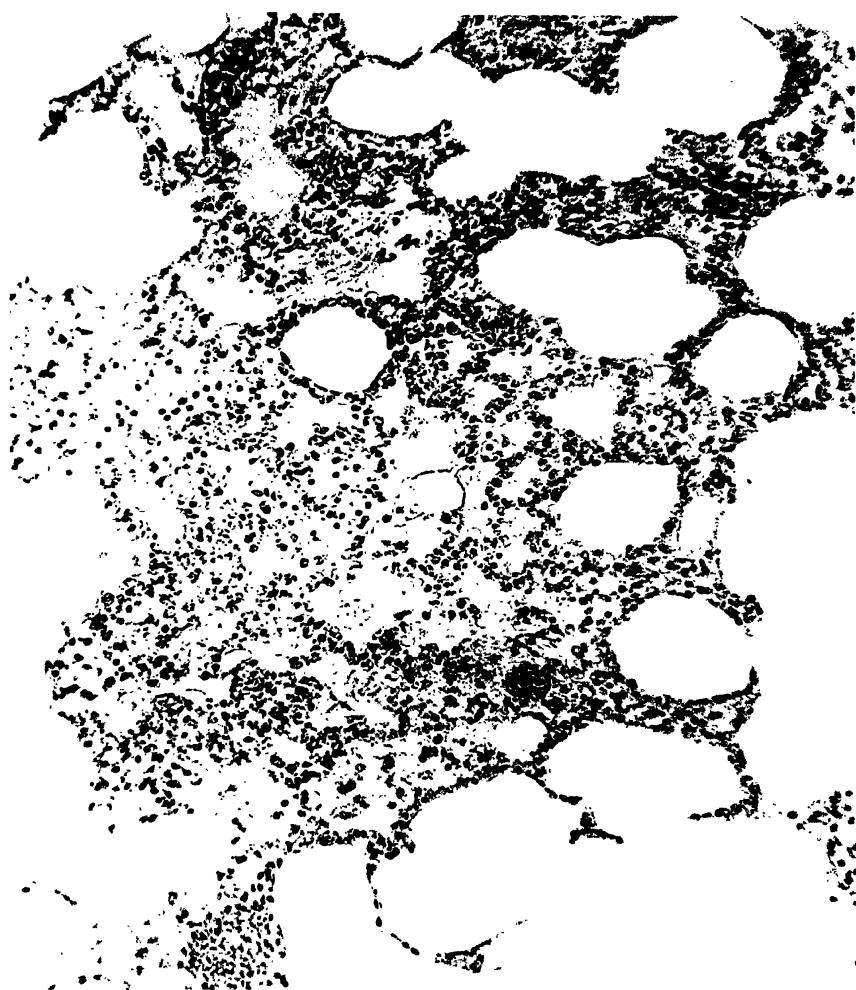
Fig. 8.

FIG. 9.

EXPERIMENTAL STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

V. BACTERIUM PNEUMOSINTES AND CONCURRENT INFECTIONS.

BY PETER K. OLITSKY, M.D., AND FREDERICK L. GATES, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 1 TO 3.

In the preceding article[1] of this series, we described the anaerobic, filter-passing organism which has been cultivated by special methods from the nasopharyngeal secretions of patients in the early hours of uncomplicated epidemic influenza, and from the lungs of rabbits and guinea pigs experimentally inoculated with these secretions. Earlier experiments had demonstrated the presence, in the nasopharyngeal secretions, of an active agent of peculiar character, distinguished from ordinary bacteria by its effects on the lungs and blood of experimental animals, by filter passage, and by resistance to glycerolation for a period of months.[2,3] Parallel experiments with the anaerobic organism disclosed a similarity of biologic properties and pathogenic effects sufficient to establish its identity with the active agent. We have, therefore, stated[1] that the pathogenic activity of the nasopharyngeal washings from early cases of uncomplicated epidemic influenza, as tested in our experimental animals, was due to the presence of the anaerobic, filter-passing organism which we have recovered from these secretions.

A peculiar and significant property of the active agent, in view of its origin, was its effect in reducing the resistance of the lung tissues of inoculated animals to accidental or experimental infection with bacteria of ordinary species—those bacteria for example which were so

[1] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 713.

[2] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 125.

[3] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 361.

frequently found in concurrent or secondary pneumonias associated with epidemic influenza in man.[4]

If the active agent in influenzal secretions has been identified in the filter-passing organism, it follows that this organism should have the same property of reducing pulmonary resistance in experimental animals. It is the purpose of the present paper to describe our observations and experiments bearing on this point.

Accidental Concurrent Infections with Ordinary Bacteria.

Under the heading of accidental infections may be grouped those scattered experiments in which the intratracheal inoculation of a washed mass culture of the filter-passing organism was followed by a pulmonary infection with ordinary bacteria. We have already described such accidents after inoculation of the active agent from influenzal washings.[4] These experiences were less frequent in the later experiments with the filter passer, due to the avoidance of oral contamination after the catheter method first employed was discarded in favor of needle puncture of the exposed trachea.[5]

Nevertheless, ordinary bacteria were encountered in the lungs of six rabbits in five series of transmission experiments with cultures originally derived from a strain of the filter-passing organism obtained from a case in the first, or 1918–19, epidemic. Three other transmission series remained uncomplicated throughout. In these experiments the first passage was initiated with a culture of the filter passer. Subsequent passages were effected with suspensions of lung tissue from the preceding rabbits. Thus the organism was carried through as many as five rabbit passages, and subsequently recovered.

Bacillus welchii was recovered from two successive rabbit passages, the fourth and fifth, of one transmission series. *Bacillus coli, Bacillus lepisepticus, Staphylococcus aureus,* and a large Gram-positive bacillus were each recovered once (Table I). The occurrence of such an accidental infection caused the termination of the series.

The presence of a concurrent bacterial infection in these rabbits was usually indicated by aggravated symptoms and by prostration

---

[4] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 373.

[5] All operations were performed under light ether anesthesia.

and death within 48 hours if the animal was not killed earlier. An intense injection or a purulent inflammation of the conjunctivæ developed. Loss of weight was marked. The blood picture showed a greater depression of the leucocytes, involving both the polymorphonuclear and the mononuclear cells than that occurring in the animals which were injected with *Bacterium pneumosintes*. In the rabbits in which *Bacillus coli* and the Gram-positive bacillus were encountered, the initial depression was followed by a polymorphonuclear leucocytosis.

TABLE I.

Occurrence of Ordinary Bacteria in the Course of Transmission Experiments with B. pneumosintes.

Generation of B. pneumosintes beginning the series.	No. of rabbit passages showing typical effects.	Rabbit passage showing secondary infection.	Kind of bacteria.	Pathological effect in rabbit.
Second.	5	Second (only in one of two rabbits).	*Staphylococcus aureus.*	Diffuse polymorphonuclear consolidation with abscesses.
Fourth.	2	Second.	*B. coli.*	Abscess of lungs.
"	2	" (only in one of two rabbits).	Large Gram-positive aerobic bacillus.	" " "
Eighth.	5	Fourth. Fifth.	*B. welchii.* " "	Bronchopneumonia. "
Ninth.	4	Fourth.	" *lepisepticus.*	Purulent bronchitis; patchy pneumonia.

At autopsy the familiar pulmonary lesions—hemorrhagic edema and emphysema without consolidation or pleuritis—were complicated or masked by other lesions attributable to the ordinary bacteria involved. A diffuse polymorphonuclear exudation was accompanied by patchy or lobar consolidation, localized small abscesses, and necrosis of the vascular endothelium with thrombus formation. A purulent bronchitis resulted from the *lepisepticus* infection. From these lesions the invading organisms were cultivated and identified.

Thus the course of accidental bacterial infection in rabbits inoculated with the filter passer closely paralleled the findings in similar infections accompanying the active agent of the earlier experiments. A more exact basis of comparison is afforded by parallel experiments with the active agent and the filter-passing organism in which concurrent or secondary bacterial infections were experimentally induced.

Experimental Concurrent Infections.

With the exception of *Staphylococcus aureus*, the accidental invaders in our experiments do not belong to the group of organisms commonly found in concurrent or secondary pneumonias so frequently associated with epidemic influenza in man. For the production of experimental concurrent infections we therefore chose two organisms as examples of the frequent inhabitants of the nasopharynx which have been recovered from many postinfluenzal pneumonias, *Bacillus pfeifferi* and a Type IV pneumococcus.

We have already described[4] the results of intratracheal or intravenous injection of these organisms alone in the small doses employed in the following experiments. Their effects were transient and differed essentially from the equally transient effects of the influenzal active agent. It was the combined action of bacteria and active agent in the same rabbit which produced the fatal pneumonias in the experimental animals and led us to point out the significant similarity of these pneumonias to those associated with epidemic influenza in man. With these earlier experiments as a basis of comparison we were now ready to study the effects of the ordinary bacteria in combination with *Bacterium pneumosintes*.

Rabbits were first inoculated intratracheally with the cultivable bodies and then received suitable doses of the chosen bacteria either intratracheally or by an ear vein.

The following protocols illustrate the results of the injection of cultivable bodies and ordinary bacteria by the intratracheal route.

Protocol 1.[6]   *B. pneumosintes and Pneumococci.*—Nov. 17, 1920. A rabbit whose normal temperature was 38.9°C., total leucocytes 11,000, of which 4,620 . were mononuclears, was inoculated intratracheally with the washed sediment of a

---

[6] Only typical protocols of a number of similar experiments are presented.

fourth generation mass culture[7] of cultivable bodies originally derived from the 1918–19 epidemic. Nov. 18. Temperature 39.3°C., total leucocytes 8,600, of which 1,892 were mononuclears. Inoculated intratracheally with one loopful of a 48 hour growth of Type IV pneumococcus on a standard agar slant. Nov. 20. Temperature 40°C., leucocytes 6,000, of which 2,400 were mononuclears (Text-fig. 1). Killed. The lungs showed lobar consolidation (red hepatization) of the right upper and lower lobes and a small patch of consolidation in the left upper lobe. The lesion in the left lower lobe consisted of edema, emphysema, and a number of small hemorrhages. Film preparations of the consolidated area showed 80 per cent polymorphonuclear cells and a few pneumococci. Aerobic cultures yielded Pneumococcus Type IV.

Control rabbits were separately injected with the same doses of *B. pneumosintes* and the pneumococcus. In the first instance the injection was followed in 48 hours by a fall in the total leucocyte count, mainly due to a drop in the mononuclears. The rabbit was killed. At autopsy the lungs showed a typical hemorrhagic edema and emphysema without consolidation. Aerobic cultures remained sterile. Anaerobic cultures yielded *B. pneumosintes* in pure culture.

The second control rabbit, injected intratracheally with one loopful of pneumococcus culture, was killed after a similar interval. The lungs showed no lesion. Aerobic cultures were free from growth.

Protocol 2. B. pneumosintes and B. pfeifferi.—Dec. 11, 1920. A rabbit whose normal temperature was 39.2°C. and total leucocytes 7,000, of which 3,850 were mononuclears, was inoculated with the washed sediment of a seventh generation mass culture of cultivable bodies originally derived from the first, or 1918–19, epidemic. Dec. 12. Temperature 39.5°C. Total leucocytes 4,600, of which 794 were mononuclears. Inoculated intratracheally with the 24 hour growth on a blood agar slant of *B. pfeifferi*. Dec. 13. Temperature 39.5°C. Total leucocytes 10,000, of which 2,500 were mononuclears (Text-fig. 2). Killed. The lungs showed pneumonic consolidation of the right and left upper lobes, and edema, emphysema, and patchy hemorrhages in the other lobes. The diffuse polymorphonuclear exudation in the consolidated areas is shown in Fig. 1. Aerobic cultures yielded *B. pfeifferi*.

A control rabbit, inoculated intratracheally with the same dose of *B. pneumosintes* showed the clinical and pathological effects regarded as typical and already described at length[1] (Fig. 2).

A second control rabbit was injected intratracheally with the washed sediment from an uninoculated control of the mass culture medium, and on the following day with the same dose of *B. pfeifferi* that was given to the experimental animal. This control rabbit was killed 24 hours later. The lungs showed no lesions. Aerobic cultures yielded no growth.

---

[7] The dose and preparation of the growth in mass culture for inoculation are described in another paper.[1]
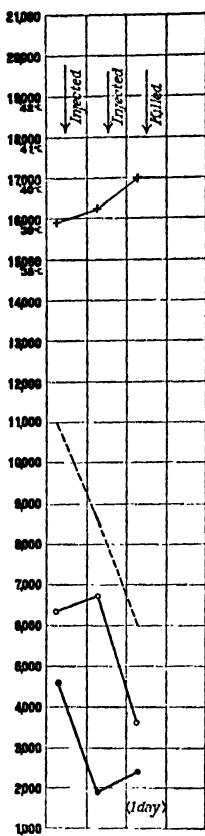
TEXT FIG. 1.   TEXT FIG. 2.
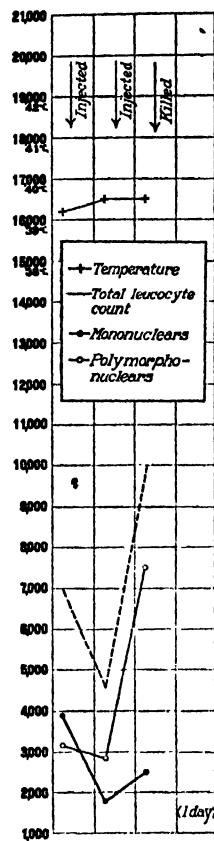
TEXT-FIG. 1. Effect on blood count and temperature (Protocol 1). The first intratracheal injection consisted of the cultivable bodies and was followed by a rise in temperature and depression of the total leucocytic count, caused by a deficiency of mononuclears. The second intratracheal injection, of pneumococci, caused no essential change in temperature or blood count.

TEXT-FIG. 2. Effect on blood count and temperature (Protocol 2). The first intratracheal injection consisted of the cultivable bodies and was followed by a rise in temperature and depression of the total leucocytic count, caused by a deficiency of mononuclears. The second intratracheal injection, of *B. pfeifferi*, caused a polymorphonucleosis.

The injection of cultures of the cultivable bodies, followed by sub-infective doses of pneumococci or Pfeiffer's bacilli, produced a lobar or bronchopneumonic consolidation of the lungs with polynuclear exudation, combined with the hemorrhagic edema and emphysema typical of the *Bacterium pneumosintes* and the influenzal active agent.

Cultures from the consolidated areas yielded profuse growths of pneumococci or *Bacillus pfeifferi*. Control rabbits injected with the cultivable bodies alone showed only the hemorrhagic edema and the emphysema, without consolidation (or polymorphonuclear cell invasion). Control rabbits injected with the ordinary bacteria, in the small doses employed, showed only a transient polymorphonuclear leucocytosis and no visible lung lesions.

These experiments, therefore, gave results similar to those observed after the intratracheal injection of the active influenzal agent and *Bacillus pfeifferi* or a pneumococcus.

The intratracheal injection of both organisms imitated, in a manner, the probable mode of infection in man. In a second series of experiments, as in the former experiments with the influenzal agent, the intravenous route for the injection of the ordinary bacteria was chosen as a more severe test of concomitant action, even though the blood stream may not be the portal of entry of the lungs in postinfluenzal pneumonias in man.

A series of rabbits was injected intratracheally with a third generation culture of the cultivable bodies, originally derived from the nasopharyngeal secretions of a case in the first epidemic (1918-19). 24 hours later they were given, by ear vein, small doses of a Type IV pneumococcus or of Pfeiffer's bacillus which proved subinfective for control animals.

Under the conditions of the experiment the ordinary bacteria showed a selective localization in the lung tissues where they set up an active infection resulting in more or less extensive lobar or bronchial consolidation, with profuse polymorphonuclear exudation and fibrin formation with necrosis of the vessel walls and thrombus production. The rest of the lung tissue showed the hemorrhagic edema and the emphysema characteristic of infection with *Bacterium pneumosintes* alone. Type IV pneumococci or Pfeiffer's bacilli were recovered from the consolidated areas.

These observations on the results of experimental concurrent infection with the cultivable bodies and ordinary bacteria closely parallel those already described[4] as the result of similar experiments with the active agent and the corresponding ordinary bacteria.

They demonstrate that *Bacterium pneumosintes* possesses the same peculiar property of lowering the threshold of resistance of the pulmonary structure to infection with ordinary bacteria. As a result subinfective doses of Type IV pneumococci and of Pfeiffer's bacilli, for example, become infective and invade the vulnerable tissue, with the establishment of such reactions as are typical of postinfluenzal pneumonia in man. Thus additional proof is afforded of the identity of the active agent of the nasopharyngeal secretions in influenza and the cultivable bodies derived from the same source, and a further parallel is drawn between the accidental or experimental production of concurrent or secondary bacterial pneumonias in animals and the frequent occurrence of similar postinfluenzal pneumonias in man.

SUMMARY.

During the course of animal experiments with the anaerobic filter-passing organisms cultivated from epidemic influenzal sources, certain pulmonary infections with ordinary bacteria have been observed. The experiments also have shown that the lungs of animals infected with *Bacterium pneumosintes* are less resistant than normal lungs to infection with ordinary bacteria. The demonstration of this fact invites a comparison of the course of these experimental bacterial infections with the sequence of postinfluenzal pneumonias attributable to similar organisms in man.

These observations furnish additional proof of the identity of *Bacterium pneumosintes* and the active agent derived from the nasopharyngeal secretions of patients in the early hours of epidemic influenza.

EXPLANATION OF PLATES.

PLATE 1.

FIG. 1. Microscopic lesions in the lungs of a rabbit described in Protocol 2, injected intratracheally with *B. pneumosintes* followed by similar inoculation in 24 hours with *B. pfeifferi*. The diffuse polymorphonuclear exudation is noteworthy. Compare with Fig. 2.   × 95.

PLATE 2.

FIG. 2. Microscopic lesions in the lungs of a rabbit described in Protocol 2, and injected intratracheally with the cultivable bodies alone. The hemorrhagic edema and emphysema are noteworthy.   × 95.

PLATE 3.

FIG. 3. Gross lesions in the lungs of a rabbit injected intratracheally with the cultivable bodies, and 24 hours later, intravenously with pneumococci. The hemorrhagic consolidation of the left lung, and the hemorrhagic edema and emphysema of the right are noteworthy. Natural size.

FIG. 4. Gross lesions in the lungs of a rabbit injected intratracheally with the cultivable bodies, and 24 hours later, intravenously with *B. pfeifferi*. The hemorrhagic and patchy consolidation of the left lung, and the hemorrhagic edema and emphysema of the right are shown. Natural size.
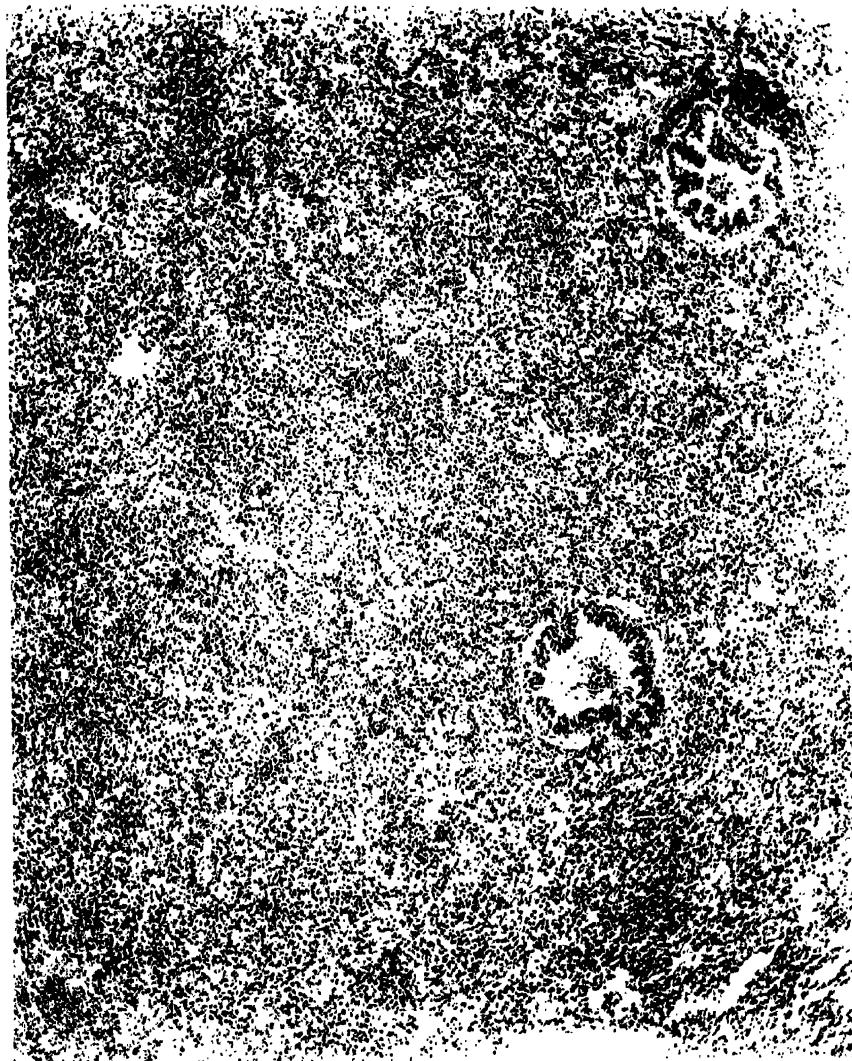
FIG. 1.
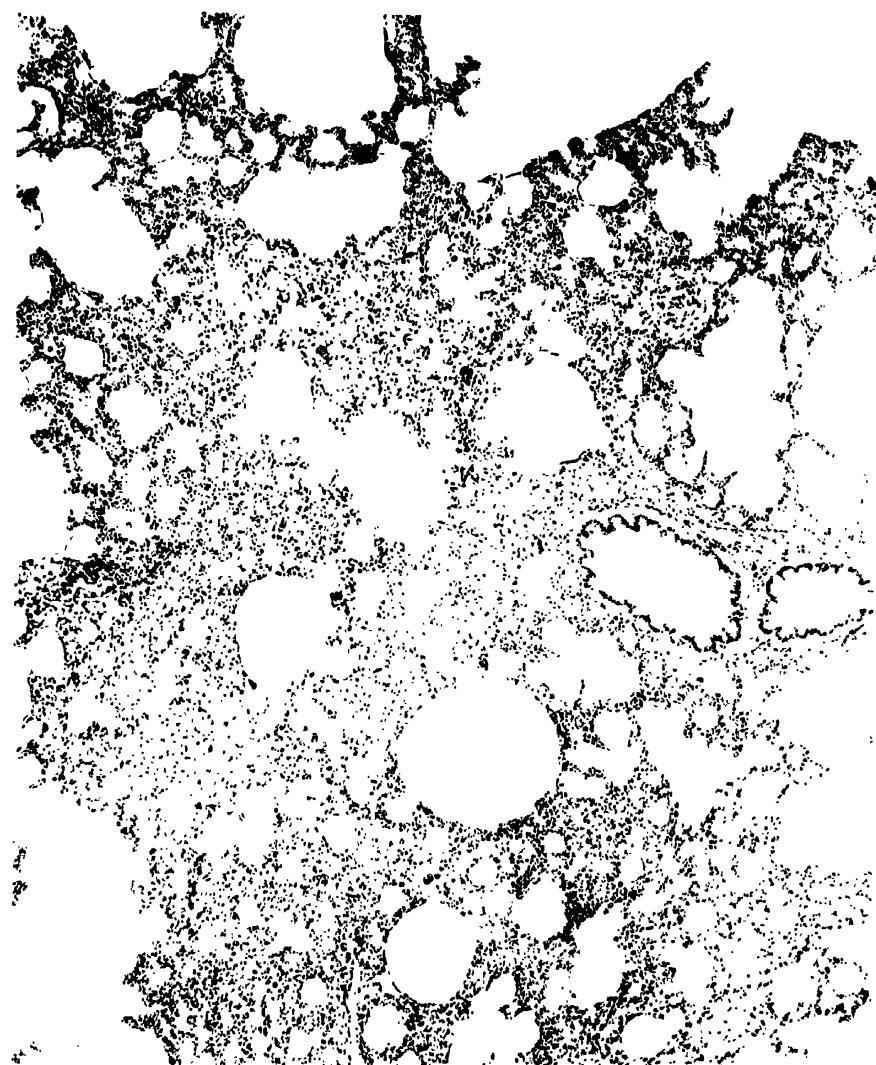
FIG. 2.

Fig. 3.

Fig. 4.

THE BILIARY OBSTRUCTION REQUIRED TO PRODUCE JAUNDICE.

BY PHILIP D. McMASTER, M.D., AND PEYTON ROUS, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

In a recent paper from this laboratory[1] observations were reported proving that a part of the rabbit's liver can function for the whole as regards bile elimination. A good instance in point is furnished by an experiment performed with another end in view. In a series of rabbits the ducts from three-fourths of the hepatic tissue were ligated and the portal branch to the remaining fourth was tied at the same operation, thus increasing the portal flow and by consequence bile formation in the mass with obstructed ducts. Under such circumstances the entire burden of bile elimination was laid on a mere quarter of the parenchyma, and this supplied with blood only by the hepatic artery, a source furnishing approximately two-fifths of the normal quantity if one may judge from data obtained in dogs.[2] Yet the rabbits remained unjaundiced and healthy. More recent observations upon dogs have convinced us that the experiment will yield a similar result with them. And it has been a surprise to discover on a search of the literature that no general recognition exists of the large margin of safety of the liver in bile elimination. On the contrary, frequent categorical statements may be found to the effect that, in man at least, jaundice is often caused by lesions affecting only a small proportion of the hepatic parenchyma.

Quincke and Hoppe-Seyler[3] state that transient obstruction of a small duct branch may bring about the absorption of sufficient bile to lead to a clinical

[1] Rous, P., and Larimore, L. D., *J. Exp. Med.*, 1920, xxxi, 609.

[2] Macleod, J. J. R., and Pearce, R. G., *Am. J. Physiol.*, 1914, xxxv, 87.

[3] Quincke, H. I., and Hoppe-Seyler, G., in Nothnagel, N., Specielle Pathologie und Therapie, Vienna and Leipsic, 2nd edition, 1912, xviii.

jaundice. The obstruction may be difficult to find at autopsy and the small ducts should be carefully searched for it. According to Eppinger[4] icterus is roughly proportional in intensity to the size of the occluded ducts, and localized inflammatory processes may cause it. Rolleston[5] believes that it follows the occlusion of one branch of the hepatic duct although the other continues to pour bile into the intestine; but not so according to Naunyn[6] who asserts that icterus under these circumstances fails to develop save when there is a complicating *Cholangie*. Krehl[7] holds that icterus occurs in many diseases through local stagnation and resorption. Recently van der Bergh and Snapper[8] have called attention to the fact that intrahepatic tumors of considerable size, and manifestly occluding many ducts, are often unaccompanied by jaundice, whence it follows in their opinion that local lesions in general must frequently exist without causing an accumulation of bile pigment in the organism.

The divergence of opinion illustrated by these specimen citations is obviously the result, first, of a lack of experimental evidence on the essential point at issue—the ability of a part of the liver to act for the whole as concerns bile elimination—and, second, of conclusions from clinical instances complicated by many factors.

For the work here to be described dogs and monkeys have been employed. Rabbits could not be used because their bile pigment fails to react satisfactorily to the ordinary tests.

Criteria of Bile Retention.

The clinician as a rule is first apprized of deficient bile elimination in his patient by the development of bilirubinuria with or without a tissue icterus. As our prime aim has been to determine how much biliary obstruction may exist without clinical manifestations, the tests in current use have been adopted for the work.

These tests as applied to the blood are very unsatisfactory. The Gmelin reaction with blood serum, so strongly advocated by French workers,[9] is yielded only when bilirubinemia is obvious to the eye, while furthermore lutein gives a

[4] Eppinger, H., *Ergebn. inn. Med.*, 1908, i, 107.

[5] Rolleston, H. D., Diseases of the liver, London, 2nd edition, 1912.

[6] Naunyn, B., *Mitt. Grenzgeb. Med. u. Chir.*, 1919, xxxi, 537.

[7] Krehl, L., Pathologische Physiologie, Leipsic, 9th edition, 1918, 571.

[8] van der Bergh, A. A. H., and Snapper, J., *Berl. klin. Woch.*, 1914, i, 1109.

[9] Gilbert, A., Herscher, M., and Posternak, S., *Compt. rend. Soc. biol.*, 1903, lv, 530; 1905, lvii, 250.

positive reaction; and the intensity of color of the plasma itself, save in outspoken cases, is trustworthy only in the absence of hemolysis and of extraneous pigments such as carotin. The reactions of Obermeyer and Popper[10] have proved in our hands little more sensitive than that of Gmelin. The diazo test recently advocated by van der Bergh and Snapper[11] is delicate, and promises to be of great clinical value, but its use has been limited as yet and it is not entirely specific.[12] We have employed it in the present work in its negative aspect, as the criterion wherewith to rule out bilirubinemia, for which it would seem highly suitable. None of many dog sera obviously stained with bile has failed to give the test, and the unstained sera have regularly proved negative.

The diazo reagent is a mixture, made fresh each day, of the following stock solutions. (a) 5 gm. of sulfanilic acid and 50 cc. of hydrochloric acid in 1,000 cc. of distilled water. (b) 0.5 per cent solution of sodium nitrite in distilled water. For use 1 part of (b) is added to 50 of (a).

The oxalated or citrated plasma to be tested is first shaken briefly with two volumes of 95 to 96 per cent alcohol and centrifuged to throw out the precipitate that forms. The supernatant fluid will now contain all of the bilirubin unless the plasma held very large quantities, in which case some will be carried down with the precipitate and must be extracted with 64 per cent alcohol if a quantitative result is to be obtained. On the addition of the diazo reagent to the fluid containing bilirubin, in the proportion of 1 part to 4, the beautiful violet tint of azobilirubin appears after a few minutes. To determine its amount a colorimeter is used and a solution of pure bilirubin in chloroform (5 mg. per 100 cc.) to give with the reagent a standard tint.

The Gmelin reaction has been used to detect bilirubinuria, and to quantitate it Hooper and Whipple's[13] modification of Salkowski's method has been employed as routine. The sodium nitrite-nitric acid solution recommended by Gilbert, Herscher, and Posternak[9] has been found to yield a better Gmelin response than the ordinary fuming nitric acid.

In contrast with the tests just mentioned, those for bile salts in the urine are none of them satisfactory. Pettenkofer's reaction was employed for one series of dogs, but Hay's sulfur reaction was adopted for most of the animals as less open to technical error and relatively specific.[14] Attempts to detect cholates in the blood were after many trials abandoned. The method recently described by Hoover and Blankenhorn[15] whereby the salts are separated out by dialysis and

[10] Obermeyer, F., and Popper, H., Wien. med. Woch., 1910, lx, 2592.

[11] van der Bergh, A. A. H., and Snapper, J., Deutsch. Arch. klin. Med., 1913, cx, 540.

[12] Koessler, K. K., and Hanke, M. T., J. Biol. Chem., 1919, xxxix, 497.

[13] Hooper, C. W., and Whipple, G. H., Am. J. Physiol., 1916, xl, 332.

[14] Lyon-Caen, L., J. physiol. et path. gén., 1910, xii, 526.

[15] Hoover, C. F., and Blankenhorn, M. A., Arch. Int. Med., 1916, xviii, 289.

TABLE I.

Bilirubinuria in the Absence of Bilirubinemia in Dogs.

Normal dogs.				Operated dogs.				
Dog No.	Procedure.	Urine.	Blood.	Dog No.	Operation.	Time of observation.	Urine.	Blood.
16	Normal dog. Fasting on 6th and 9th to 12th days inclusive.	Bile pigment present on 6th and 8th to 14th days inclusive.	Negative.	19	All ducts ligated but those to lateral liver mass; 71 per cent of liver obstructed.	Before operation.	Bile pigment present for 3 days.	Negative.
						After operation.	Bile pigment present on 1st to 8th, and on the 10th day after operation.	Pigment in plasma on 6th, 7th, and 8th days after operation.
17	Normal dog. Fasting on 6th and 9th to 14th days inclusive.	Bile pigment present on 6th, 7th, 9th, and 11th days.	"	8	Ducts to lateral mass left free; 71 per cent of liver obstructed.	Before operation.	Bile pigment present for 5 days.	Negative.
						After operation.	Bile pigment present on 2nd, 3rd, and 12th days.	"
18	Normal dog. Fasting on 6th day only.	Bile pigment present on 6th and 7th days.	"	10	Ducts to left lateral lobe left free; 72.4 per cent of liver obstructed.	Before operation.	Negative.	"
						After operation.	Bile pigment present on 4th, 5th, 10th, and 12th days.	"

12	Duct to right lateral lobe left free; 82 per cent of liver obstructed.	Before operation. After operation.	Negative. Bile pigment present on 4th, 9th, and 11th days.	Negative. " until 11th day, then faintly positive.
11	Duct to right lateral lobe left free; 82 per cent of liver obstructed.	Before operation. After operation.	Negative. Bile pigment present on 6th day.	Negative. "

concentrated failed regularly in our hands to give positive results with the plasmas of jaundiced dogs excreting cholates through the kidneys. This may have been due to the lack in the dog of a renal threshold for bile salts such as in man leads to their accumulation in the circulation.

The Renal Threshold for Bilirubin.

In man, as is well known, the normal blood plasma has a yellow color owing to the presence of bilirubin; and a considerable increase in the pigment may occur without the passage of any into the urine, much less the appearance of a tissue icterus.[15] According to van der Bergh and Snapper[11,16] the plasma of healthy dogs is colorless and this has been our own finding except in animals with "physiological jaundice" induced by fasting. The plasma of normal monkeys is also in our experience practically colorless and fails to give the diazo reaction. We have further noted that in both dogs and monkeys bilirubinemia never occurs without bilirubinuria, while in dogs, at least, bile pigment is frequently to be found in the urine when it cannot be demonstrated in the circulation (Table I).

The state of the urine, then, furnishes a more delicate criterion of icterus in the dog than does that of the blood. The conditions are very different in man. In man, owing to the high renal threshold for bilirubin a considerable accumulation of pigment takes place in the blood before any escapes into the urine, and consequently one must reckon from a base-line of normal pigment retention in work upon defective bile elimination. This complicating factor does not exist in the dog and monkey. Such a renal threshold as is present in these animals—and *a priori* one would be expected on grounds of biological relationship—has so slight an elevation as to produce no evidence in the blood of its presence.

Method.

The animals were kept in metabolism cages and the 24 hour urines were examined for a number of days prior to operation, as regards their general character and the presence of bile salts and pigments. The monkeys were males, but most of the dogs chosen were females and with one exception (No. 10) none was taken which

---

[16] van der Bergh, A. A. H., and Snapper, J., *Berl. klin. Woch.*, 1914, li, 1109.

showed spontaneous icterus, so called. Throughout the work the routine tests were carried out upon cage urines, but positive findings were controlled by catheterization. At more or less frequent intervals, as occasion warranted, the blood was examined for bilirubin. The monkeys were fed on bananas, the dogs on bread and meat.

The biliary obstruction was induced by ligating and, where possible, cutting various branches of the hepatic duct under ether anesthesia. Asepsis was maintained, and the wound in the abdominal wall closed in three layers. When the free portion of the duct to be obstructed was too short to be doubly tied and cut, several stout ligatures were laid upon it, a procedure which in the dog generally served to close the duct throughout the term of experiment. Indeed, we have been but little troubled with the restoration of the duct channels by the cutting through of ligatures, possibly because of the large caliber of the silk used. In monkeys, on the other hand, even coarse ligatures were found to work through the duct walls within 10 days to 2 weeks, and either the continuity of the channel was reestablished, or a leak from it led to death. Needless to say, at every autopsy a careful study was made of the final results of operation and cultures were taken from the liver and the stasis bile. The animals were killed with chloroform. Instances in which infection existed were ruled from consideration.

Arrangement of the Dog and Monkey Livers.

The main liver mass of the dog consists of the left lateral and central and right central lobes with the ill defined quadrate lobe as an essential part of the last which also bears the gall bladder on its under surface.[17] There is another mass, separate both by contour and tissue cleavage, the lateral mass as we shall term it, which lies just above and to the right of the pylorus and is made up of the right lateral and caudate lobes. The main liver contains on the average about six-tenths of the parenchyma, the lateral mass about three-tenths. The remaining one-tenth consists of the papillary, or Spigelian, lobe placed below the main liver and separated from the lateral mass by the gastrohepatic omentum, but connected with both by tissue bridges. The course of the bile ducts falls in roughly with the anatomical arrangement of the tissue which they drain. A large branch coming from the left lateral and central lobes joins another from the right central and caudate lobes,—into which, by the way, the cystic duct opens,—and thus there is formed a large main channel into which the small papillary branch empties, and, lower down, within a few centimeters of the intestine, the considerable duct from the lobe mass. Frequent marked variations from this typical arrangement are encountered. Thus, for example, the branch from the lobe mass may course toward the main liver to join that from the right central and caudate lobes. Or the branch which appears to spring from the whole left central and

---

[17] Bradley, O. C., A guide to the dissection of the dog, London, New York, Bombay, and Calcutta, 1912.

TABLE II.

Weight of the Lobes of the Normal Dog Liver, Expressed in Percentages of the Total Organ.

Dog No.	Body weight.	Sex.	Liver weight.	Papillary lobe.	Lateral mass.		Entire lateral mass.	Main liver.			Entire main liver.
					Right lateral.	Caudate.		Left lateral.	Left central.	Right central.	
	kg.		*gm.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	10	M.	315.2	5.8	19.5	9.8	29.0	25.4	11.5	26.5	65.2
2			181.5	7.3	14.8	8.7	29.3	32.9	16.4	22.1	63.4
3	7¼	M.	226.0	5.1	16.8	14.8	23.5	24.5	13.6	24.2	71.4
4	11	F.	330.5	6.1	22.8	13.2	31.6	27.1	12.1	20.2	62.3
5	15	M.	545.0	4.6	12.4	8.6	36.0	30.1	15.8	27.7	59.4
6	11	F.	305.5	5.4			21.0			19.1	73.6
7	7	M.	212.5	4.7			34.0				61.3
8	11¾	"	505.0				40.0				60.0
9	8	F.	279.0				27.0				73.0
10	9¼	M.	288.0	6.2			22.7				77.3
11	11	"	337.0				23.4			28.4	70.4
12	11¼	F.	423.0				27.1	37.0			72.9
13	8	M.	253.5				21.0	27.2			79.0
14	9	"	333.5				25.8				74.2
15	7	F.	242.0				31.0				69.0
16	7	M.	335.0				20.0				80.0
17	8	F.	359.0	5.0			33.2			21.8	61.8
18	10¼	M.	359.5	4.6			32.0				63.4
19	9	F.	351.0	6.0			27.1	25.0			66.9
20	8¾	"	335.0	4.9			25.3	27.7			69.8
21	7	"	231.5	4.1			21.6	28.5			74.3
22	12¾	M.	472.0	4.9			32.6	25.8	18.6	27.2	62.5
23	6	F.	285.0	4.6			36.8	24.5			58.6
24	8	"	252.5	3.9	21.6	14.5	36.1	27.7	11.7	20.6	60.0
25	6½	"	235.7	4.4	19.5	9.5	29.0	29.7	11.0	25.9	66.6

26	F.	193.0	5.2	18.1	12.5	30.6	26.4	11.9	25.9	64.2
27	M.	261.5	4.6	22.8	14.7	37.5	26.8	11.1	20.0	57.9
28	"	311.0	5.8	15.4	12.2	27.6	25.0	9.5	32.1	66.6
29	F.	138.5	5.1	15.8	13.8	29.6	25.9	13.1	26.3	65.3
Average			5.1 (21 cases).	18.1 (11 cases).	12.0 (11 cases).	29.0 (29 cases).	27.6 (18 cases).	13.0 (12 cases).	24.5 (15 cases).	67.3 (29 cases).
Variation			3.9–7.3	12.4–22.8	8.6–16.9	20.0–40.0	24.5–37.0	9.5–18.6	19.1–32.1	57.9–80.0

lateral lobes may in reality drain but a small portion of these, their bile emptying for the most part into the just mentioned duct from the right central and caudate lobes. Large vasa aberrantia are frequent. They may be patent and filled with. bile for several centimeters above their junction with the common duct, but stop short of the liver tissue or cease thereabouts to be patulous. A mistaken dependence upon such vessels for bile elimination has in some of our experiments led to the development of total obstruction when a partial one had been projected.

The liver lobes are in general defined by a cleavage of the parenchymanearly to the hilum of the organ; and when there is but one duct from a lobe or group of lobes its obstruction leads to stasis throughout the tributary region. The proportion of the total liver affected can be readily determined under such circumstances. Potentially, at least, the case is different when there are two ducts or more to a lobe and one is left open. Under such circumstances relief may perhaps come to the area in stasis through newly opened channels into the unobstructed neighboring tissue. For it is well known that the bile canaliculi anastomose freely within the lobules. That no relief comes of a magnitude meriting consideration will be shown further on. The main difficulty lies in determining the exact amount of tissue in stasis.

Table II gives the weights of the liver lobes of twenty-nine normal dogs, expressed in percentages of the organ. In all cases the liver was removed before the blood had clotted. It will be seen that there is a rather large individual variation in the tissue distribution.

The monkey liver is divided into five lobes, much as in the rabbit, and these are grouped into a main liver and a lateral mass, which are connected at the base by a broad tissue bridge, as are the individual lobes also. A single short duct comes from each of the masses mentioned, and these unite to form an hepatic duct into which the cystic duct enters lower down, as in human beings. None of the ducts from the individual lobes save that from the caudate is accessible to ligation. This circumstance like that of the early cutting through of the ligatures has much hampered our observations.

"Physiological Jaundice."

Investigators upon icterus in the dog agree that it is frequently encountered in mild form in animals that appear normal. Naunyn[18] showed, as far back as 1869, that fasting for 24 hours regularly leads in most dogs to the appearance of bile pigment and salts in the urine. The icterus is not dependent on increased concentration of the urine, though it is made more evident thereby. A bilirubinuria from fasting was a frequent complicating factor in our early experiments, occurring

[18] Naunyn, B., *Arch. Anat., Physiol. u. wissensch. Med.*, 1869, 579.

regularly in the Sunday to Monday 24 hour specimen of unoperated animals as a result of the small ration of the day first mentioned. When a full diet was provided on Sundays intercurrent icterus became rare and often was traceable to disease. It was never found as the result of anesthesia or of the trauma of operation. In monkeys a fasting icterus was not noted, although it is said to develop occasionally in human beings.[3]

In view of these facts it seemed wise to keep several normal animals under observation with the operated ones as a control to intercurrent manifestations, and this was accordingly done.

Results of Total Obstruction in the Dog.

In sixteen dogs total obstruction was produced, sometimes by ligating and severing the common duct, but more frequently by cutting its large tributaries after their individual ligation. The tissue icterus that followed was never as pronounced as in human beings under similar conditions but in all cases was easily recognizable, while, in all, bile pigment became abundant in the blood and urine.[19] The tissue icterus was first visible on the 5th to 10th day, as yellowed scleras. The results in four of the sixteen dogs which were followed with special care have been tabulated (Table III).

The first sign of biliary obstruction was the appearance, as a rule, of pigment in the urine during the second or third 24 hours after operation but sometimes earlier. The delay is due, as Affanassiew showed,[20] to bile accumulation in the gall bladder and distended ducts: when he filled these reservoirs with wax at the time of operation icterus developed very much more promptly. Bilirubinemia was usually noted within 24 hours after bilirubinuria, and cholates were recognizable in the urine on the 3rd or 4th day. At autopsy of the dogs, after 9 to 46 days of obstruction, a general tissue jaundice was regularly found, the liver being especially affected.

[19] We have since autopsied an animal in which after 9 days of total obstruction no tissue icterus was discoverable. Urine and plasma were markedly tinted with bilirubin. There is little doubt that tissue pigmentation would have occurred in a day or so more.

[20] Affanassiew, M., Z. klin. Med., 1883, vi, 281.

TABLE III.

Results of Total and Partial Obstruction in Dogs.

Dog No.	Sex.	Findings prior to operation.	Observed after operation for. *days*	Weight of animal. Initial. *kg.*	Weight of animal. Final. *kg.*	Liver portion and per cent obstructed.	Postoperative findings. Bilirubinemia.	Postoperative findings. Urine.	Remarks.
1	F.	Negative for 9 days.	26	10¾	11	Total obstruction.	Positive on 3rd day and after.	Pigment on and after 2nd day; salts on 3rd day and thereafter.	Scleral jaundice appeared on 8th day.
2	"	Negative for 10 days.	15	9½	9½	"	Positive on 2nd day and after.	Pigment on and after 2nd day; salts on 5th day and thereafter.	Scleral jaundice appeared on 5th day.
3	"	Negative for 7 days.	20	3¾	2½	"	Positive on 4th day and after.	Pigment and salts positive on 3rd day and after.	Scleral jaundice appeared on 8th day.
4	"	Negative for 4 days.	13	8¾	8½	"	Positive on 3rd day and after.	Pigment and salts on 3rd day and after.	Scleral jaundice appeared on 9th day.
5	"	Negative for 3 days.	26	10¼	10¾	All except lateral mass; 71 per cent.	None.	Negative.	
6	"	Negative for 4 days.	10	9½	9½	"	"	" save for salts on 3rd day.	
7	"	Negative for 7 days.	37	7¼	6¼	"	"	Negative.	
8	"	Slight bilirubinuria of unknown cause during 5 days.	36	7¼	8¼	"	"	Pigment and salts faintly positive on 2nd and 3rd days; salts positive on 16th, 20th, and 21st days.	Unoperated control animals showed pigment and salts likewise.

No.	Sex	Pretreatment				Thyroid removed	Bile in urine	Notes	Controls
9	F.	Negative for 6 days.	23	5¾	5¼	All except left lateral lobe; 72.4 per cent.	None.	Pigment dubiously positive on two occasions.	Unoperated control animals showed pigment and salts likewise.
10	M.	Negative for 7 days.	34	17	16	"	"	Pigment occasionally throughout.	
11	"	Negative for 6 days.	36	14¾	16¼	All except right lateral lobe; 82 per cent.	"	Salts from 7th to 14th days; thereafter, like pigment, negative.	Pigment once dubiously positive in urine.
12	F.	Negative for 7 days.	45	15¾	14¼	"	"	Salts from 8th to 37th days; pigment on 4th, 9th, and 42nd days.	
13	"	Negative for 7 days.	15	9½	9½	All except papillary lobe; 95 per cent.	Positive on 11th day and after.	Pigment on 2nd, 3rd, and 4th days; salts and pigment on 11th day and thereafter.	
14	"	Negative for 7 days.	15	6¾		"	Positive on 2nd day and after.	Pigment on 2nd day and after; salts on 1st day and after.	
15	F.	Negative for 8 days.	28			Control animal.	None.	Pigment on 18th and 20th days; pigment and salts on 22nd, 24th, and 25th days.	
16	"	Negative for 4 days.	33			"	"	Negative throughout.	
17	"	Negative for 5 days.	32			"	"	Pigment on 11th, 12th, 13th, and 14th days; salts on 14th day.	
18	"	Negative for 5 days.	30			"	"	Pigment and salts on 6th day and pigment on 19th day.	Pigment once dubiously positive in urine.

Results of Partial Obstruction in the Dog.

Partial obstruction was produced in ten dogs. Bile accumulation within these animals would presumably take place more slowly than on total obstruction, owing to the smaller amount of tissue in stasis and to the eliminative activities of the unobstructed portion. For these reasons no animal was considered to have given negative results as regards icterus until at least 10 days after operation and most were kept under observation for several weeks. As we shall point out further on, the unobstructed liver portion undergoes some hypertrophy within 10 days, while that in stasis atrophies, and thus the tendency to bile retention must soon be counteracted by lessened secretion in the region of stasis combined with increased facilities for elimination.

In four dogs approximately seven-tenths of the liver parenchyma was placed in stasis, in two others about three-fourths, in two, four-fifths, and in two about nineteen-twentieths. Table III summarizes the findings. Tissue icterus is not recorded because it was never observed, even when the eliminative burden had been thrown abruptly upon a mere twentieth of the liver.

In the four animals in which the duct from the lateral mass, draining on the average 29 per cent of the whole organ, was alone left open, bile pigment and salts were never demonstrable in blood or urine save on days when they were also present in control animals from the same intercurrent cause, namely, fasting. This was the case too when the free duct was that from the left lateral lobe containing 27.6 per cent of the tissue. A slightly greater degree of obstruction, produced by tying and cutting all the ducts except that to the right lateral lobe, or 18 per cent of the liver, resulted in the appearance of bile salts in the urine during the 2nd week after operation, occasionally accompanied in one animal by bile pigment as shown with the Gmelin test. The amount of pigment was always too slight to be quantitated, and bilirubinemia was not observed. Finally, when all the ducts were closed except that from the little papillary lobe, which holds from 3.8 to 7.3 per cent of the liver tissue, or on the average 5.1 per cent, pigment and cholates did indeed appear regularly in both blood and urine, and almost as rapidly as when total obstruction had been

produced. In the two instances studied a sufficient bile elimination took place, however, to prevent tissue icterus during the 15 days of observation, and in view of the papillary hypertrophy found at the end of this period there is but slight reason to suppose that a greater retention would have occurred later.

The four normal dogs that shared the general conditions of the operated animals and were followed in the same way yielded findings that were several times of great value, disclosing that bilirubinuria and choluria noted in the operated animals were "physiological," from food deprivation. In this connection it seemed of interest to determine whether animals with seven-tenths of the liver in bile stasis would show fasting icterus more readily than normal controls. Accordingly during a period of 6 days several animals of each sort were fed only a thin bouillon. This they took in quantity with result that the urinary output remained large. The experiment will be set forth in detail in a later paper on the physiology of jaundice. Here we shall merely state that while icterus appeared in most of the animals, it developed no sooner and was no more marked in the operated individuals than in the controls.

Liver Adaptation.

In dogs with total biliary obstruction jaundice of the liver parenchyma is outspoken after a few days. By contrast, when obstruction is partial, even when it affects nineteen-twentieths of the organ, an hepatic icterus is not observed. From this it is evident that vicarious bile elimination becomes effective very close to the source, so to speak, a fact which is not surprising when one considers that the hepatic cells lie in the midst of copious blood and lymph streams, which, if kept free of bile, should keep them free also.

Long continued obstruction to the ducts from a part of the canine liver results in noteworthy changes in the whole organ, just as in the rabbit[21] and in man.[22] The portion in stasis gradually becomes sclerotic by an interlobular proliferation of the connective tissue, and the parenchymal cells undergo a gradual simple atrophy, and may

---

[21] Nasse, *Verhandl. deutsch. Ges. Chir.*, 1894, xxiii, pt. 2, 525.

[22] Carnot, P., and Harvier, P., *Arch. méd. exp. et anat. path.*, 1907, xix, 76.

TABLE IV.

Results of Total and Partial Obstruction in Monkeys.

Monkey No.	Previous findings.	Observed after operation for	Weight of animal.		Liver portion and per cent obstructed.	Postoperative findings.		Remarks.
		days	Initial.	Final.		Bilirubinemia.	Bilirubinuria.	
			gm.	gm.				
1	Negative for 13 days.	7	1,900	1,900	Total obstruction.	Positive on 1st day and after.	Positive on 1st day and after.	Tissue icterus on 4th day and after.
2	Negative for 1 day.	8	2,375	2,300	"	Positive on 1st day and after.	Positive on 1st day and after.	Tissue icterus on 3rd day and after.
3	Negative for 1 day.	11	2,300	2,375	"	Positive on 2nd day and after.	Positive on 2nd day and after.	Tissue icterus on 3rd day and after.
4	Negative for 1 day.	12	3,100	2,900	"	Positive on 5th day and after.	Positive on 5th day and after.	At operation a pathological dilatation of the bile passages was noted. Tissue icterus on 8th day and after.
5	Negative for 5 days.	12	2,300	2,325	All except lateral mass and papillary lobe; 75 per cent.	None.	None.	Duct continuity restored toward end of experiment.
6	Negative for 21 days.	11	1,975	2,025	All except lateral mass; 80 per cent.	"	"	Questionable restoration of duct's continuity toward end of experiment.
7	Negative for 5 days.	11	2,650	3,225	" "	"	"	Obstruction maintained.

ultimately disappear. Meanwhile, the tissue with duct unobstructed gradually hypertrophies. The underlying causes for these alterations have been analyzed in a previous paper from this laboratory.[1] In the dog they take place far more slowly than in the rabbit. A parenchymal shift is usually not discernible in less than 10 days, but after a month may be very marked. At this time the mass in stasis may be much shrunken, with finely hobnailed surface. Needless to say, instances free of infection are here alone referred to. The region with obstructed bile channels is now sharply demarcated from the adjacent hypertrophic tissue, and has been found to correspond closely in extent with the ramifications of these channels, thus proving that during stasis no important connections open between the blocked ducts and the neighboring free ones.

The shifting of tissue caused by local obstruction renders it impossible to determine exactly from the weights of the liver portions at autopsy how much of the parenchyma was originally placed in stasis. But the anatomical relations and a knowledge of the normal proportions of the liver lobes, such as Table II affords, enable one to reach an approximate conclusion on this point.

Results of Biliary Obstruction in the Monkey.

Seven monkeys were used (Table IV). They yielded results essentially similar to those in the dog. As in this animal, total obstruction was well tolerated, but bilirubinuria appeared more rapidly, developing in two out of four instances within the first 24 hours after operation. What would seem to be an interesting illustration of delay in its appearance owing to accumulation of bile in the ducts and gall bladder is afforded by Monkey 4. At operation a note was made that the bile passages of this animal, though undistended, were about three times the usual diameter. Despite the production of total obstruction, pigment failed to appear in urine or blood until the 5th day and tissue icterus was not seen until the 8th day. At autopsy all of the bile passages were enormously distended.

The plasma of monkeys subsisting on a banana diet is colorless and the urine nearly so. Bilirubinemia appears at approximately the

same time as bilirubinuria, on the 1st or 2nd day of total obstruction, and tissue icterus follows on the 3rd or 4th day. Cholates were never demonstrable in the urine by Hay's method. Further work would seem desirable on this point.

In three monkeys 75 to 80 per cent of the liver was placed in stasis. Owing to the rapidity with which ligatures cut through the ducts it was deemed best to terminate the experiments 11 and 12 days after operation. Even then in one instance there had been partial restoration of the duct continuity with some escape of bile into the intestine, though the well defined anatomical changes in the liver indicated that this was recent. At no time did the blood or urine of any of the animals contain bilirubin. That this would have continued to be the case is shown by the autopsy findings. For the livers, even in so brief a time, had undergone marked alterations in the direction of a functional readjustment. The unobstructed tissue was notably hypertrophied, and that in stasis shrunken and sclerotic. The changes had taken place almost as rapidly as in the rabbit, far more so than in the dog.

DISCUSSION.

The ability of a small portion of the liver to function for the whole as regards bile elimination when there is local obstruction depends without doubt upon several factors. That the parenchymal cells can rapidly excrete bile pigment and salts coming to them in large amount on the blood stream is proven by the promptness with which these substances pass into the bile when thrown into the circulation for experimental purposes.[23] Bile constituents formed by the tissue in stasis, and carried away from it by the blood and lymph, will of course be treated similarly. But the conditions of stasis themselves tend to lessen the amount of bile formed. For the obstructed ducts, dilating under pressure, interfere with the local portal flow[24] and divert a portion of the blood and with it the functional activities, to the more normal hepatic regions. There follows in due course a pa-

[23] Wertheimer, E., *Arch. physiol. norm. et path.*, 1892, iv, series 5, 577. Stadelmann, E., *Deutsch. med. Woch.*, 1896, xxii, 785.

[24] Betz, W., *Sitzungsber. k. Akad. Wissensch. Math.-naturw. Cl., Wien.*, 1862, xlvi, 238.

renchymal shifting from the region in stasis,[1] which, as time passes, tends to become complete. It is possible, furthermore, that stasis directly influences the liver cells to form less bile, but this cannot be profitably discussed.

Attempts should be made to determine in human beings the margin of safety in bile elimination. Quantitative studies on the rate of disappearance of jaundice after the surgical relief of total biliary obstruction should provide interesting figures on the ability of the liver to excrete pigment in excess. Injections post mortem into the bile ducts to determine the degree of obstruction in congenitally cystic livers and livers with widespread carcinomatosis ought, in connection with the clinical findings, to yield data of value. Perhaps the most direct evidence, though, is to be had from cases of local obstruction by intrahepatic calculi.

Beer,[25] who described many instances of stones within the liver, remarks on the fact that even when numerous and widely distributed they often cause no symptoms during life. Lewisohn[26] tells of finding at operation a liver studded with fibrous nodules in each of which was one or more gall stones. Yet the patient had for a long time been in good health and unjaundiced. Carnot and Harvier[22] report the complete atrophy of a liver lobe as result of an intrahepatic calculus occurring in a patient who was never jaundiced. Such instances, as well as those more frequent ones of disseminated hepatic carcinomatosis without jaundice, which come under clinical observation, give good reason for the belief that the human liver possesses a margin of safety in bile elimination not inferior to that of the dog and monkey.

There is a point of immediate practical import in the demonstration that the biliary obstruction required to produce jaundice is one affecting the greater portion of the liver. Jaundice is not infrequently seen in association with abscesses and other less discrete inflammatory changes occupying but a small portion of the hepatic tissue. The inference from such observations has been that the jaundice results from local bile resorption. But in view of our

[25] Beer, E., *Med. News*, 1904, lxxxv, 202.
[26] Lewisohn, R., *Ann. Surg.*, 1916, lxiii, 535.

findings this would seem highly unlikely. Rather should one think in such instances of a general injury either to the liver parenchyma or ducts, or else to the blood corpuscles.

SUMMARY.

The bile ducts from three-quarters of the liver substance in dogs and monkeys can be obstructed without any clinical evidence developing of pigment or cholate accumulation in the organism. And in the dog nineteen-twentieths of the liver substance can be placed in stasis without the occurrence of tissue icterus such as regularly follows total obstruction in this animal. There is no reason to suppose that this will not be found true in the monkey as well. Always a local obstruction results sooner or later in atrophy of the affected tissue with compensatory hypertrophy elsewhere. Thus as time passes the derangement of function produced by the sudden stasis is progressively lessened.

The plasma of the dog and monkey, unlike that of man, is normally free from bilirubin, and this pigment so readily escapes from the blood into the urine that bilirubinuria is often to be found in the dog in the absence of bilirubinemia, while the latter is never met with alone in either animal. It follows that in both species the renal threshold for bilirubin is much lower than in man,—if indeed one can be said to exist at all.

The amount of biliary obstruction required to produce jaundice in human beings is probably as great as in the experimental animals with which we have dealt. The clinical jaundice encountered in association with local liver lesions should be viewed not as the result of local bile resorption, but as due to a general injury to the hepatic parenchyma or ducts, or to blood destruction.

DISSOCIATION OF MICROBIC SPECIES.

I. Coexistence of Individuals of Different Degrees of Virulence in Cultures of the Bacillus of Rabbit Septicemia.

By PAUL H. De KRUIF, Ph.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

INTRODUCTION.

It is a general rule to regard the individuals comprising a given strain of a microbic species as identical in nature. So, when a culture is spoken of as virulent, it is supposed to be virulent as a whole, and little or no attention is paid to its individual members. They are tacitly assumed to possess this character in common. As a culture attenuates by passage on artificial media, the formerly virulent germs are again assumed to lose their invasive property simultaneously and uniformly.

This point of view persists in spite of numerous facts that show the assumption of a community of characteristics to be without foundation. Chauveau and Phisalix (1) demonstrated atypical forms of *B. anthracis* in cultures from the lymph glands of animals that had died of this infection. Their *bacille en clou* possessed a morphology distinctly different from that of the typical organism. It was completely avirulent. Elser and Huntoon (2) found that colonies of different appearance existed side by side in freshly isolated cultures of *Micrococcus catarrhalis,* and that one type, increasing with each passage, tended finally completely to supplant the other. Bordet and Sleeswijk (3) found an interesting variation to occur in the behavior of *B. pertussis.* When freshly isolated, this organism grew only upon media enriched with blood, but after several passages it became able to multiply upon ordinary media. The blood microbe had antigenic characters distinctly different from those of the agar microbe. Von Lingelsheim (4) discovered that variants having a peculiar type of colony formation arise in laboratory cultures of *B. typhosus.* His work has been elaborated and

extended to other members of the colon-typhoid group by Gildemeister (5). Baerthlein (6) has demonstrated the simultaneous presence, in single cultures of *B. pyocyaneus*, *Staphylococcus aureus*, and *B. coli*, of individuals giving rise to colonies of different appearance. The "H" and "O" forms of *B. proteus*, first described by Weil and Felix (7), are well known. Eisenberg (8) and Wagner (9) have studied thoroughly the presence of variants in cultures of *B. anthracis*.

The researches of Bordet on phagocytic reactions *in vivo*, following injections of *Vibrio metchnikovii* (10) and of streptococcus (11), have brought out with striking clarity differences in virulence existing among the individuals of one culture. It is remarkable that little stress has been laid on findings of such significance.

With this exception, differences of virulence have received little attention in the above mentioned studies. It has been our good fortune, in the course of studies on the rabbit septicemia bacillus, to observe wide variations in this characteristic, associated with definite difference in growth on fluid media and in colony formation. If all the organisms in these cultures had possessed indistinguishable growth characters, little progress could have been made. As it is, the association of a readily observable criterion with the virulence variation has greatly simplified the study of this problem.

Origin of Materials.

The strains of the rabbit septicemia bacillus used were isolated from spontaneous infections occurring among the normal animal stock of this laboratory. Eight strains were obtained in pure culture from the heart's blood at necropsy. The pathologic picture in six of these cases was that of bronchopneumonia, fibrinopurulent pleuritis, and pericarditis. In the remaining two no gross lesions of any kind were discernible, but the organisms were present in large numbers in the heart's blood.

Method of Isolation.

The heart's blood obtained at necropsy was planted in 10 per cent defibrinated rabbit blood broth. After incubation for 24 hours the cultures were plated out in 5 per cent rabbit serum agar. Isolated colonies were fished to rabbit serum broth. After incubation, growth, and microscopic study, this culture was streaked on 10 per cent rabbit serum agar slants, incubated for 10 hours, and then stored in the ice

chest for 7 days, when it was again transplanted. This routine was rigorously observed for all strains.

Characteristics of the Organism.

The organism under investigation is without doubt one of the pasteurella. It is probably identical with the bacillus of rabbit septicemia first accurately described by Theobald Smith (12). It is a minute non-motile bacillus, occurring singly or in pairs. It is frequently coccoid in shape and in fresh preparation might be mistaken for pneumococcus. It is Gram-negative when stained by Stirling's method. Its bipolar nature is best demonstrated in films from the heart's blood, fixed by heat, and stained for 5 minutes in the cold with Loeffler's methylene blue. The microbes in the films from pleural fluid, tissues, and cultures tend to stain solidly. Pleomorphism is most marked in the condensation water of serum or plain agar slants.

Beef infusion broth and beef infusion agar, pH = 7.4, fractionally sterilized, were used as base medium throughout the investigation. All the strains are strongly aerophilic. When first isolated from spontaneous infections they grow very feebly or not at all on ordinary media, but quite abundantly on serum agar or in serum broth. After several transplants on blood or serum agar, good growth occurs on ordinary media, provided that a sufficient quantity of material is carried over. The surface colonies on agar are circular in shape, with even borders. They are whitish and rather opaque at the center, more translucent toward the edges. They are strongly fluorescent, both in daylight and by artificial illumination. In serum and in plain broth the growth is uniformly turbid with little tendency to sedimentation. In tubes of serum broth, inoculated with 0.05 cc. of a 16 hour culture, the lag period is very short and the peak of growth reached by 8 hours incubation. From that time on a rapid decrease in the number of viable organisms occurs.

There is no visible growth on potato. Carbohydrates do not enhance growth. The strains isolated in this laboratory ferment dextrose, levulose, saccharose, and xylose with acid but no gas. The medium used was sugar-free broth to which 1 per cent of carbohydrate was added. The pH before inoculation was 7.5. After incubation at 37°C. for 66 hours, the levulose, dextrose, and saccharose tubes were found to have reached an acidity of pH = 5.9 to 6.1. No acid formation was observed in maltose or lactose. In fact, definite alkalinity increase was observed with lactose—pH = 8.2. Three strains received through the kindness of Dr. N. S. Ferry behaved as did those just described, with the exception that maltose was fermented.

In the course of virulence studies with strains isolated about 6 weeks previously, the approximate number of organisms injected into the test rabbits was controlled by plating high dilutions of the test culture. Dilutions corresponding to 10^{-9}, 10^{-8}, and 10^{-7} cc. of the serum broth cultures under test were plated in 5 per cent rabbit serum agar. It was thought that plain agar might serve as well for this procedure, since all the strains under test had been growing for some time in artificial media. But upon seeding such dilutions

TABLE I.

Growth of Dilutions of Serum Broth Culture R 15 in Serum and Plain Media.

Dilution of original culture.	No. of colonies on plain agar plate.	No. of colonies on serum agar plate.	Growth in plain broth.	Growth in serum broth.
cc.				
10^{-3}	0	Countless.	—	—
10^{-4}	0	"	—	—
10^{-5}	0	"	+	+
10^{-6}	0	1,500	0	+
10^{-7}	0	194	0	+
10^{-8}	0	21	0	+
10^{-9}	0	2	0	+

+ indicates growth; 0, no growth; —, culture not made.

into plain agar plates, no growth occurred, despite the fact that animals injected with the same dilutions succumbed with typical infections. It was known that good growth could be obtained upon plain agar when a transplant was made of fairly large amounts from tube to tube. It was considered of importance to find out how many organisms it would be necessary to plant in order to obtain growth in medium without serum.

Experiment 1. Growth of Dilutions of a 12 Hour Serum Broth Culture in Serum and Plain Agar and Serum and Plain Broth.—A 12 hour 5 per cent rabbit serum broth culture of Strain R 15 was diluted with plain broth, pH = 7.4, to 10^{-9} cc. of the original culture. The dilutions were made as follows: Dilution A, 1 cc. of culture + 4 cc. of broth; Dilution B, 0.5 cc. of Dilution A + 4.5 cc. of broth; Dilution C, 0.5 cc. of Dilution B + 4.5 cc. of broth; and so on, to Dilution I, 0.5 cc. of which would be equivalent to 10^{-9} cc. of the original. Different pipettes

were used for making each dilution.   As soon as the dilutions were completed, and starting at dilution 10^{-9} cc., 0.5 cc. was carried over into parallel large tubes of plain and of 10 per cent horse serum agar, which had been melted and cooled to 45°C.   The tubes were carefully shaken and the contents poured at once into plates.   Such plates were made from all dilutions, from 10^{-9} down to 10^{-3} cc. At the same time, inoculation of the same amount of material was made into parallel series of serum and of plain broth tubes, from dilutions 10^{-9} to 10^{-5} cc. The plates and tubes were incubated at 37°C. for 48 hours.   The result is given in Table I.

Table I shows that no growth occurred in plain agar plates, even when these were seeded with 10^{-3} cc. of the culture, an amount containing millions of organisms.   Colonies appeared on all the serum agar plates, and in all the serum broth tubes.   On the other hand, the plain broth series showed multiplication in 10^{-5} cc., but not in higher dilutions.   The most striking fact observed was the character of the growth in the plain broth subculture from dilution 10^{-5} cc.   Instead of growing diffusely with a uniform turbidity, as was the case with all the serum broth subcultures, the growth in the plain broth tube was granular in character.   These granules were apparent even in early phases of the growth.   They settled rapidly to the bottom of the tube, so that in 24 hours the supernatant fluid was perfectly clear.

Repetition of this experiment led to the same result.   This phenomenon can be explained in one of two ways.   First, it might be conceived that organisms growing diffusely in serum broth subcultures might flocculate when cultivated in plain broth.   Or, second, two types of microbe might be supposed to exist in the same culture, one, preponderatingly present, and capable of growing only in serum broth, when seeded in small amount, the other, less numerous, sedimenting in fluid medium, and able to grow in plain broth, even when seeded in relatively small amount.   To resolve this question the following experiment was undertaken.

Experiment 2. Persistence of the Growth Character of Granular and Diffuse Growing Microbes.—The plain broth subculture, dilution 10^{-5} cc., of Experiment 1 grew in rapidly sedimenting, granular manner.   This culture was seeded in amounts of 0.05 cc. to parallel tubes of plain and of 5 per cent rabbit serum broth.   The procedure was repeated through six transfers.   The granular growth persisted, in the serum as well as in the plain broth tubes.   On the other hand,

it will be recalled that the serum broth subculture, dilution 10^{-9} cc., of Experiment 1, exhibited a diffuse growth. This culture, seeded in similar amount into parallel serum and plain broth tubes, continued to grow diffusely in the plain as well as in the serum broth series.

It would seem, then, that the first of the two possibilities suggested, *i.e.* that of a change of growth character following transfer from serum to plain broth, is answered in the negative. On the other hand, the possibility still remained that the sudden transfer from serum to plain broth might cause a mutation from diffuse to granular growth character, which might persist even after return to serum broth. To rule out this contingency, it was necessary to determine other differences between the granular and diffuse growing microbes, which might lead to the demonstration of their existence side by side in the same culture; that is to say, it was necessary to reveal their coexistence in a given stock culture, without having recourse to dilution into plain broth.

Experiment 3. Difference in Appearance of Colonies of the Granular and Diffuse Growing Microbes.—A stock serum agar slant of Strain I' 15 was subcultured into 5 per cent rabbit serum broth. The culture after 6 hours showed diffuse growth. It was diluted to 10^{-8} cc. in plain broth, and subinoculations were made as before into parallel series of plain and serum broth tubes. The serum broth subculture showed diffuse growth in dilutions 10^{-8} to 10^{-2} cc. The plain broth parallel subcultures grew in dilutions up to 10^{-6} cc., but not in dilutions 10^{-7} or 10^{-8} cc. All the tubes of this series were granular in their growth character.

The serum broth subculture, 10^{-8} cc. (diffuse), and plain broth subculture, 10^{-6} cc. (granular), were now transferred into 5 per cent serum broth. The transplant from the first grew diffusely and was designated D. That from the second sedimented rapidly; it was designated G. Cultures D and G were again carried over to serum broth. After 6 hours incubation at 37°C., each tube was diluted in plain broth in the usual manner to 10^{-8} cc. of the original. Tube G was thoroughly shaken to break up the clumps before dilution.

Dilutions 10^{-7} and 10^{-8} cc. of Cultures D and G were plated in 5 per cent serum agar. The surface colonies resulting from Inoculation D were strikingly different from those of Inoculation G. The former were whitish, with rather opaque, glistening centers, fading into translucent outer zones. Their borders were regular. They exhibited marked fluorescence, both by daylight and by artificial light. The surface colonies of Type G were somewhat smaller, translucent, and bluish in color, had irregular serrated edges, and showed little or no fluorescence.

Colonies of the first type, D, fished to 5 per cent serum broth, yielded diffuse growth. Those of Type G gave rise in every instance to granular, rapidly sedimenting cultures.

The fact that the difference in appearance of colonies accompanied the difference in growth character in fluid medium was utilized at once as a means of detecting whether Types G and D exist side by side in strains which have never been carried into plain broth. It will be seen by reference to Table I that the Type G organisms in the original culture are present in smaller numbers than those of Type D. Since, ordinarily, Type G grows in plain broth subcultures from dilutions not higher than 10^{-4} cc., it will be evident that there is no hope of detecting Type G colonies by plating out high dilutions of serum broth cultures. Consequently, the demonstration of coexistence had to be sought by another method.

It was observed that when stock serum agar cultures of the various strains were planted in serum broth the resulting growth was diffuse, but that after 8 to 10 hours incubation there was always a more or less abundant sediment at the bottom of the tube. It was assumed that if the granular organisms were present with the diffuse variety, it would be logical to find them in high concentration in the sediment. This assumption was tested in Experiment 4.

Experiment 4. Demonstration of the Coexistence of Types G and D by Plating the Supernatant Fluids and Sediments of Serum Broth Cultures.—A stock 10 per cent serum agar slant of Strain R 22 was transferred to a tube of 5 per cent rabbit serum broth. After incubation at 37°C. for 24 hours, the tube contained a uniformly turbid supernatant fluid, and in addition a distinct sediment. The supernatant fluid was carefully drawn off by a Pasteur bulb pipette, care being taken not to disturb the sediment. The supernatant fluid was placed in a sterile tube, Tube A.

The sediment was now thoroughly shaken up in 10 cc. of sterile broth—Tube B. Tubes A and B were streaked upon plain and serum agar plates, which were then incubated for 24 hours. The result is summarized in Table II.

The two different type colonies were now fished from the serum agar plates to serum broth. Subcultures from the translucent, non-fluorescent type invariably yielded granular strains. Those from the opaque, fluorescing colonies gave rise to an invariably diffuse growth. Experiment 4 demonstrates clearly that Type G and D microbes

coexist in the stock serum broth tubes.   It shows that Type G organisms, usually in the minority, can be easily demonstrated by the separation of the supernatant fluid and the sediment of a serum broth culture, and the streaking of the latter.   It must be pointed out in this place that this method of procedure is only necessary when Type D greatly predominates.   It has been found that when cultures of the rabbit septicemia bacillus are infrequently transplanted, the more saprophytic Type G tends to gain the upper hand.   Under these conditions simple streaking out of a thoroughly shaken culture suffices to place both varieties in evidence.

TABLE II.

Dissociation of Types G and D by Sedimentation.

Tube.	Material.	5 per cent serum agar plate.	Plain agar plate.
A	Supernatant fluid of 24 hr. serum broth culture, Strain R 22.	Good growth; both types present. Type D (fluorescent) preponderates.	Scanty growth; both types present, but Type D preponderates.
B	Sediment of 24 hr. serum broth culture, Strain R 22.	Good growth; both types present. Type G (non-fluorescent) largely preponderates.	Very scanty growth; almost entirely non-fluorescent Type G.

Up to the present three differential characteristics of Types D and G have been discussed.   (1) Type G is able to grow much more easily than Type D in plain broth; that is to say, much larger amounts of Type D than of Type G are necessary to cause multiplication in this medium.   (2) Type G grows in minute granules in liquid media, these particles sedimenting rapidly and leaving the supernatant fluid clear.   Type D, on the other hand, grows diffusely, both in serum and in plain broth.   (3) The colonies produced by Microbe D are distinctly different from those of Type G.

Careful study has revealed no difference in morphology or tinctorial reaction between Types G and D.   Their fermentation reactions have proved to be identical.   The question arises whether other differences exist.

Since Microbe G grows with distinctly greater ease in plain broth, it is easy to imagine that this fact might represent an adaptation of the organism, ordinarily rather delicately adjusted to the parasitic state, to a saprophytic existence; that is to say, Type G, which is more saprophytic, might exhibit a corresponding loss in parasitic activity, and hence might be of decidedly less virulence for rabbits than the corresponding Type D.

This hypothesis is found to be vindicated in a striking manner in Experiment 5.

TABLE III.

Comparative Virulence of Types G and D, Strain R 15.

Type.	No. of passages after dissociation.	Weight of rabbit.	Age of culture.	Amount injected intra-pleurally.	Result.
		gm.	*hrs.*	*cc.*	
D	Serum broth third passage.	550	6	10^{-7}	Negative.
		550		10^{-6}	Died in 42 hrs.*
		550		10^{-5}	Died in 42 hrs.*
G	Plain broth first, serum broth second passage.	550	6	10^{-7}	Negative.
		550		10^{-6}	"
		550		10^{-5}	"

* Autopsy typical; fibrinopurulent pleuritis, pericarditis, and bronchopneumonia.   Pure culture of Type D from heart's blood.

Plate of dilution 10^{-8} cc., Type G, 32 colonies.

Plate of dilution 10^{-8} cc., Type D, 21 colonies.

Experiment 5. Comparative Virulence of Types D and G.—Microbe D was obtained by transplanting a diffuse growing serum broth subculture from dilution 10^{-8} cc. of a 6 hour serum broth culture of Stock Strain R 15.   Third passage in serum broth.   Microbe G was obtained by transplanting a granular plain broth subculture from dilution 10^{-6} cc. of the same serum broth culture of Strain R 15.   Passage in serum broth exactly parallel to that of Microbe D.

Serum broth cultures of each variety were incubated for 16 hours and seeded in 0.05 cc. amounts into tubes of 5 per cent rabbit serum broth.   Incubation for 6 hours at 37°C.   Dilution in plain broth to 10^{-8} cc., care being taken to disintegrate thoroughly the granules of Culture G.   Culture D was then injected intrapleurally, in doses of 10^{-7}, 10^{-6}, and 10^{-5} cc., into rabbits of 550 gm. weight.

Culture G was injected by the same route into rabbits of the same weight in doses of 10^{-7}, 10^{-5}, and 10^{-3} cc. Dilutions of 10^{-8} cc. of each culture were plated in 5 per cent serum agar. The result, which was most striking, is recorded in Table III.

Repetition of this experiment with the same and with other strains yielded constantly a like result. In a word, Microbe G, recently dissociated from a stock strain, shows itself to be distinctly less virulent than its companion Type D of the same strain. All experiments of this type were made with organisms dissociated from the

TABLE IV.

Virulence of Types D and G, Strain R 22, Dissociated by the Sedimentation Method.

Type.	Strain.	No. of passages in serum broth.	Age of culture.	Amount injected intrapleurally.	Result.
				cc.	
G	R 22	7	6 hrs., 1,100,000,000 colonies per cc.	10^{-4}	Negative.
				10^{-3}	"
				10^{-2}	"
				10^{-1}	"
D	R 22	7	6 hrs., 1,140,000,000 colonies per cc.	10^{-6}	"
				10^{-5}	Died in 16 days.*
				10^{-4}	Died in 4 days.*
				10^{-3}	Died in 39 hrs.*

* Autopsies in all cases typical. Pure Type D recovered from heart's blood of each animal.

same strain, at the same time, and transplanted under rigidly parallel conditions. The results in Table III are by no means the most striking obtained, since young rabbits frequently resist 0.1 cc. of a 6 hour culture of Type G, while parallel Type D cultures are often fatal in dose of 10^{-7} cc. and never in less than 10^{-4} cc.

It might be objected in the experiment just described that the Type G organisms had lost their virulence by reason of their transplantation, for one passage, in plain broth. The method of dissociation by sedimentation described in Experiment 4 makes it easy to

examine into the validity of such an objection. This technique was therefore applied to Strain R 22, and the virulence of the resulting Type G and D varieties tested. In this case Strain G had received no passage through plain broth.

Experiment 6. Virulence of Types D and G, Strain R 22, Dissociated by the Sedimentation Method.—In order to insure the purity of each type, three successive platings in serum agar, with alternate transfers into serum broth, were made, after the primary separation by sedimentation. After the third plating, Microbes D and G were carried for three passages, daily transfer, in serum broth. Each was then subinoculated from a 12 hour serum broth culture into a tube of 5 per cent rabbit serum broth. Incubation at 37°C. for 6 hours; dilution, as usual, to 10^{-7} cc. of the original culture. Type G was then injected into 1,500 gm. rabbits, intrapleurally, in doses of 10^{-4}, 10^{-3}, 10^{-2}, and 10^{-1} cc. Type D was injected by the same route into rabbits of the same size, in doses of 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} cc. The result is given in Table IV.

It must be stated here that the infection in rabbits of large size is identical in nature with that of young animals, the sole difference being in the time of death.

Experiments 5 and 6 show clearly that Microbe G is distinctly less virulent than Microbe D. It was natural to ask whether Microbe G could produce typical infections when injected in overwhelming quantities. It was found that adult rabbits tolerated doses of 0.5 cc. of whole serum broth culture. Young rabbits were for this reason selected for the experiment.

Experiment 7. Effect on Young Rabbits of Massive Doses of Type G.—A series of 600 gm. rabbits was injected intrapleurally with 3, 2, 1, 0.5, and 0.1 cc. of a 6 hour culture of Strain R 15, Type G. The rabbit injected with 3 cc. succumbed in 5 days. Those that received 2 and 0.5 cc. died in 32 and 24 hours, while doses of 1 and 0.1 cc. produced no lethal effect. It is important that the organisms recovered at necropsy showed a granular growth in serum broth, and when streaked upon serum agar plates gave rise to typical Microbe G translucent colonies.

The pathologic effects produced by Microbe G as compared with those produced by the more virulent Microbe D will be dwelt upon in a later communication in which the possibility of raising the virulence of Type G will be discussed. It is enough to say that this microbe retains its character of growth after one passage through the animal body.

It is frequently remarked that the virulence of many organisms is lost more quickly by cultivation in ordinary media than in media enriched with serum or blood. Observations have shown that with Type D (virulent) of various strains of the organisms under consideration, the characteristic diffuse growth is retained despite many passages in plain broth. Since it has been noted that the characters of diffuse growth and virulence in this instance accompany one another, it was considered important to discover whether plain broth passage would depress the virulence of Microbe D, while the diffuse growth character remained. Experiment 8 supplies an unequivocal answer to this question.

Experiment 8. Comparative Virulence of Type D, Strain R 15, after Twenty-Five Parallel Passages in Plain and Serum Broth.—Microbes D and G were dissociated from Stock Strain R 15 by the first of the methods described above; *i.e.*, by dilution and parallel subculture in plain and serum broth. They were plated three successive times in serum agar, isolated colonies being fished to serum broth each time. This insured as nearly as possible that the strains arose from a single organism. Microbe D was then carried in parallel passage through twenty-five daily transplantations in plain and in serum broth. From time to time the cultures were streaked on serum agar plates. With both the plain and the serum broth tubes organisms of Type D only were invariably found. What is more, the plain broth strain retained perfectly its characteristic of diffuse growth. The plain broth passage strain is designated as D–Pl, that of the serum broth passage as D–S.

Microbe G, dissociated at the same time, was subjected to a similar number of passages in serum broth. The virulence of all three of the strains was now tested. 6 hour serum broth cultures of Microbes D–S, D–Pl, and G–S were diluted in plain broth to 10^{-7} cc. of the original cultures. The injection of the test animals was carried out immediately after the dilution of each culture. At the end of the injections plates were in each instance made in 5 per cent serum agar. The result is summarized in Table V.

Table V shows very clearly that plain broth passage has little or no effect upon the virulence of Microbe D. On the other hand, Microbe G characteristically fails to kill in 0.1 cc. Up to the present time we have been unable to discover Type D varieties possessing low virulence in any of our strains. What is more, it is a remarkable fact that Strains R 19 and R 21, which so far have shown no evidence of organisms of Type G, are by far the most invasive of any of the

strains studied. This experiment, together with other observations described above suggest that the mechanism of attenuation may be a selection; that is, an overgrowth of the virulent Type D by the less virulent Type G. This hypothesis is amenable to experimental proof and will be discussed fully in a later communication.

TABLE V.

Comparative Virulence of Type D, Strain R 15, after Twenty-Five Parallel Passages in Serum and Plain Broth.

Type.	Strain.	No. of passages.	Weight of rabbit.	Age of culture.	Amount injected intra-pleurally.	Result.
			gm.	*hrs.*	*cc.*	
D	R 15	Serum broth, twenty-five passages.	700	6	10^{-6}	Died in 24 hrs.†
			725		10^{-5}	" " 36 " †
			650		10^{-4}	" " 42 " †
			675		10^{-3}	" " 38 " †
D	R 15	Plain broth, twenty-five passages.	700	6	10^{-6}	" " 36 " †
			750		10^{-5}	" " 24 " †
			750		10^{-4}	Negative.‡
			700		10^{-3}	Died in 31 hrs.†
G	R 15	Serum broth, twenty-five passages.	700	6	10^{-4}	Negative.
			700		10^{-3}	"
			650		10^{-2}	"
			675		10^{-1}	"

* Microbe G–S 25, control.

† Autopsy typical; fibrinopurulent pleuritis, pericarditis, and bronchopneumonia. Pure cultures of Type D from heart's blood.

‡ Probably technical error in inoculation.

Number of colonies in 10^{-7} cc.: Microbe D–S, 30; Microbe D–Pl, 53; Microbe G–S, 10.

Immunologic Relations of Types G and D.

Microbes G and D in all instances so far studied differ in growth character in fluid media, in colony formation, and in virulence. It is logical to inquire into their immunologic relations. The first experiment consisted in determining the degree of resistance offered by animals which had survived injection with Microbe G to injections of multiple lethal doses of the virulent Type D.

Experiment 9. Resistance of Animals Which Had Survived Injection with Type G to Multiple Lethal Doses of Type D.—Rabbits 1 and 2, weighing 1,500 gm. each, withstood with no visible effect 0.1 and 0.5 cc. of Type G, Strain R 15, injected intrapleurally.   14 days later they were injected by the same route with 10^{-4} cc. of a 6 hour serum broth culture of the virulent Type D, Strain R 15.   Immediately afterward, controls of the same weight received doses of 10^{-6}, 10^{-5}, and 10^{-4} cc. of the same culture.   The result is summarized in Table VI.

As seen from Table VI, Rabbits 1 and 2 were resistant to at least 100 lethal doses of the virulent Strain D.   It is of interest that such solid immunity should be conferred by a single injection of the less virulent Strain G.   Other experiments gave the same result.   It

TABLE VI.

Resistance of Animals Which Had Survived Injection with Type G, Strain R 15, to Multiple Lethal Doses of Type D, Strain R 15.

Rabbit No.	Amount of Type G injected intrapleurally 14 days previously.	Weight of rabbit.	Amount injected for test of Type D.	Result.
	cc.	*gm.*	*cc.*	
1	0.1	1,500	10^{-4}	Negative. '
2	0.5	1,500	10^{-4}	"
3		1,450	10^{-6}	Died in 9 days.*
4		1,500	10^{-5}	" " 4 " *
5		1,475	10^{-4}	" " 4 " *

* Autopsy typical.   Pure Type D recovered from heart's blood.
Number of colonies on serum agar plate of dilution 10^{-8} cc., 16.

must be remarked that no experiments have been instituted that would indicate the comparative immunizing efficacy of the virulent Type D and the less virulent Type G.

Agglutination Reactions of Types G and D.

The vaccinating power of Type G against the virulent Type D has been clearly demonstrated in Experiment 9.   The results of agglutination studies bear out the community of antigenic quality suggested by the vaccination experiments.   Stable suspensions of Microbe G (granular) may be prepared by washing the organisms from 5 per cent serum agar slants four times in distilled water, and

finally suspending them in this medium. 0.1 per cent formaldehyde was added to all suspensions.

The flocculation of both Types G and D by immune sera is very slow and is much more clear-cut at 55° than at 37°C. The necessity of incubation at 55° is especially marked with Type D. This fact is, indeed, of differential value, since Type G suspensions agglutinate very well when placed in contact with immune serum at 37°C. for 16 hours. Parallel tests made on Microbe D at the same temperature do not give satisfactory results.

Serum prepared by three intravenous injections of Microbe D into rabbits agglutinates Types D and G in titer of 1:2,000 after incubation at 55° for 16 hours. A serum resulting from similar injection of Microbe G is considerably less active, agglutinating Type G in 1:1,000 and Type D in 1:200. In some instances Microbe D agglutinated in dilution of 1:50 but not in higher dilutions. To sum up, the antigenic power, as far as the production of agglutinin is concerned, appears to be decidedly stronger in the case of Type D than of Type G.

Absorption tests clearly indicate the community of antigenic character of the two types. Serum > Type D, Strain R 15, agglutinated Microbes D and G in titer of 1:1,500. After 2 hours contact at 55° with a suspension of Microbe D, the titer for Type G had fallen to 1:200, for Type D to 1:80. The control serum, after 2 hours at 55° without the suspension, showed the original titer, 1:1,500. The same serum, placed for a similar length of time in contact with Microbe G, dropped in titer from 1:1,500 to 1:40 for Types D and G. Various observations make it apparent that Microbe G, in addition to being more easily flocculable, also has greater binding power for the agglutinating principle than has Microbe D. From the foregoing results it is apparent that there is no qualitative difference in the antigenic nature of Types G and D. In a later communication these agglutination reactions will be dealt with more fully, and the acid flocculability of the two types will be discussed. The acid agglutination points of Types D and G are distinctly different. They are of the nature of physical constants, and hence present a valuable differential criterion.

SUMMARY AND CONCLUSIONS.

Two types of organism have been shown to exist in cultures of the bacillus of rabbit septicemia, recently isolated from spontaneous infections.

One, Microbe D, grows diffusely in serum and plain broth, forms rather opaque, fluorescing colonies on serum agar, and is highly virulent for rabbits. These characters are retained throughout many passages in serum or plain broth.

The other type, Microbe G, flocculates rapidly in fluid media, forms translucent, bluish colonies with little fluorescence, and exhibits extremely low virulence for rabbits. Like Microbe D, its distinguishing characters persist throughout many passages in artificial media.

Two methods for the dissociation of these varieties from the parent culture have been described.

The two types are morphologically indistinguishable and possess identical fermentation reactions.

Rabbits surviving inoculation with Type G are resistant to multiple lethal doses of Type D. The agglutination reactions bear out this suggestion of the antigenic identity of the varieties. Community of antigenic character is rendered certain by the results of absorption reactions.

Microbe D, in contact with immune serum, flocculates well at 55°, but poorly or not at all at 37°C. Microbe G, on the other hand, agglutinates easily at both temperatures.

Microbe D, after being carried through twenty-five passages in serum and in plain broth, retains perfectly its characteristics of diffuse growth and of virulence, in the plain as well as in the serum broth.

BIBLIOGRAPHY.

1. Chauveau, A., and Phisalix, C., *Compt. rend. Acad.*, 1895, cxx, 801.
2. Elser, W. J., and Huntoon, F. M., *J. Med. Research*, 1909, xx, 370.
3. Bordet, J., and Sleeswijk, J. G., *Ann. Inst. Pasteur*, 1910, xxiv, 476.
4. von Lingelsheim, *Centr. Bakt., 1te Abt., Orig.*, 1913, lxviii, 577.
5. Gildemeister, M., *Centr. Bakt., 1te Abt., Orig.*, 1916, lxxviii, 129.
6. Baerthlein, *Centr. Bakt., 1te Abt., Ref., Beiheft*, 1912, liv, 178.

7. Weil, E., and Felix, A., *Wien. klin. Woch.*, 1917, xxx, 1509.
8. Eisenberg, P., *Centr. Bakt., 1te Abt., Orig.*, 1912, lxiii, 305.
9. Wagner, G., *Centr. Bakt., 1te Abt., Orig.*, 1920, lxxxiv, 386.
10. Bordet, J., *Ann. Inst. Pasteur*, 1892, vi, 328.
11. Bordet, J., *Ann. Inst. Pasteur*, 1897, xi, 177.
12. Smith, T., *J. Comp. Med. and Surg.*, 1887, viii. 24.

THE CONCENTRATING ACTIVITY OF THE GALL BLADDER.

BY PEYTON ROUS, M.D., AND PHILIP D. McMASTER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

The experiments here to be reported were undertaken as the result of observations upon stasis bile collected after ligation of the common duct. The accumulation of pigment in such bile seemed to us to indicate that some part of the duct system possesses a concentrating faculty of considerable moment for pathological processes.

It is current knowledge that bladder bile is normally thicker than the secretion as it comes from the liver. The point finds a brief mention in text-books. But the significance accorded it may be judged from the fact that for quantitative studies of the bile from day to day the gall bladder is regularly utilized as a link in the fistulous channel connecting the hepatic duct with the body surface.

Maly[1] states that liver bile from the dog has 3.5 to 4.9 per cent of dry substance, and that from the bladder over 20 per cent. The latter fluid, according to Hoppe-Seyler's analyses which are quoted by Maly *in extenso*, yields far the greater quantity of bile salts. Brand[2] found 1 to 4 per cent of solids in the fistula bile of human beings, and as much as 20 per cent in the bladder contents. According to Hammarsten[3] a part of the water of the bile is abstracted and a mucinous nucleoprotein added by the bladder. It is probable that this organ empties itself only partially upon contraction, and the secretion may remain in it as in a sort of backwater to be acted upon over long periods of time. Because of uncertainty as regards these matters, comparative analyses such as those just given bear but obliquely on the problem of the rapidity of the changes undergone by the bile.

In the present paper we shall deal solely with the influence of the gall bladder upon the bile, reserving for an accompanying one the influence of the ducts.[4]

[1] Maly, R., in Hermann, L., Handbuch der Physiologie, Leipsic, 1881, v, pt. 2, 172.

[2] Brand, J., *Arch. ges. Physiol.*, 1902, xc, 491.

[3] Hammarsten, O., Lehrbuch der physiologischen Chemie, Wiesbaden, 8th edition, 1914, 411.

[4] Rous, P., and McMaster, F. D., *J. Exp. Med.*, 1921, xxxiv, 75.

Method.

The best method of study will be one whereby a bile of known constitution is supplied through the normal channels to an intact gall bladder by the animal's own liver. It is practicable in the dog owing to the arrangement of the ducts. By means of a single ligature appropriately placed, a type sample of bile can be diverted for separate collection, while the remainder flows to the bladder.

The common duct of the dog is formed as a rule by the union of three large channels, and high up into the middle one the gall bladder empties. That on the right hand is derived from the caudate and right lateral and central lobes. By its entrance a few centimeters above the duodenum the common duct is finally formed, the other channels uniting much nearer the liver. If their derivative duct is tied just above where this final duct enters, all of the bile from the major portion of the hepatic tissue is pent up and directed into the gall bladder, whereas the secretion from the caudate and right lateral lobes still reaches the common duct and may be collected through a cannula. Here, in essence, is the plan of our experiments.

Owing to duct anomalies, the partition of bile effected by a ligature placed as described may vary considerably. We have made careful dissections at autopsy, tracing out each duct and ultimately determining by weight the amount of tissue delivering bile to either side of the ligature. The liver of the dog is so deeply cleft that usually this can be done accurately. But when a single lobe has ducts running to both sides of the ligature, as not infrequently happens, the partition of the tissue can be only approximately learnt.

Vigorous dogs with a wide costal angle were chosen. Under ether, a sufficient dissection was made to tie a small glass cannula into the common duct and to place higher up the essential, or partitioning, ligature, as we shall henceforth term it. In our first attempt a considerable segment of duct was freed and the gastrohepatic omentum subjected to trauma, with result that almost no bile was secreted in the 24 hours immediately following. Warned thereby, we handled the tissues of the later animals with great circumspection and met no other instances of the sort.

The bile taken as a type sample of the liver output was collected into a rubber balloon which was connected with the common duct by a short cannula and a soft rubber tube 4 to 8 cm. long and of about 2 mm. bore. The cannula was bound down into line with the duct—which was merely slit, not severed—by a thread about its shank and the lower duct portion, and thus obstruction from a kink or elbow was rendered unlikely. The balloon was left within the peritoneal cavity, and the abdominal wall closed completely in three layers. Asepsis was maintained throughout. The dogs bore the operation well, and remained in excellent condition throughout the term of experiment which was usually 24

hours. Often the animals ate largely soon after operation. All were ultimately chloroformed and immediately autopsied. Cultures were taken of the bile specimens, and pieces of liver from the regions separately drained were placed on agar and in bouillon. Infection was rare, despite the fact that the bile accumulated at body heat. Its occurrence is noted in the tables.

The pigment content of the bile was used as the index to concentration by the bladder. The sojourn of the bile for some hours at the temperature of the animal was found by repeated *in vitro* test to be without effect on the pigment, save for the conversion of a negligible portion into biliverdin when air was present. To prevent this change it was only necessary to deflate completely the collecting balloons prior to their introduction. Routine colorimetric estimations were made by Hooper and Whipple's[5] modification of the Salkowski method, whereby bilirubin and biliverdin are estimated together; but instead of a color wedge of artificial constitution, we have employed as a standard pure bilirubin, and in place of the Autenrieth instrument, a Duboscq micro-colorimeter. The bilirubin (Schuchardt) was in chloroform solution, 1 mg. to every 4 cc.; and, to prepare a standard, 1 cc. of this was made up in a volumetric flask to 10 cc. with Hooper and Whipple's acid alcohol, and allowed to stand 18 to 24 hours at room temperature, when the characteristic blue-green color described by these authors was found to have developed. The biles were treated likewise save that 1 cc. was made to 50 cc. with the alcohol. The bladder contents was often so concentrated as to necessitate some preliminary dilution with water.

Bile treated with the acid alcohol did not always go through the same color changes. Often the tint ultimately developed for the readings tended somewhat to the green, as compared with the standard, or again was pronouncedly more blue. The normal bladder bile removed at operation frequently yielded a gamut of purples, possibly as a result of the presence of bilicyanin,[6] and could at no time be read against the standard. This never happened with specimens obtained after operation. Fortunately for our work, the biles obtained from different portions of one liver during the same period always went through identical color changes and in consequence could be accurately compared; while such errors in quantitation as were involved in reading them against a standard of different tint were applicable to them in like degree. The results given in our tables are expressed in milligrams of bilirubin save for six early instances, in which the standard proved faulty. For them "color units" are employed instead. But "color units" could have been used to report the findings throughout, since the main significance of these latter lies in relative not in actual pigment quantity.

For a standard in the early experiments, a chloroform solution containing only 5 mg. of bilirubin per 100 cc. was employed, the stock solution advocated

[5] Hooper, C. W., and Whipple, G. H., *Am. J. Physiol.*, 1916, xl, 332.

[6] Hoppe-Seyler, F., in Hirschwald, A., Handbuch der physiologisch- und pathologisch-chemischen Analyse, für Aerzte und Studierende, Berlin, 8th edition, 1909, 371.

by van der Bergh and Snapper[7] in connection with the diazo reaction. A measured portion was dried on the water bath and the residual pigment taken up in acid alcohol. But it dissolved with a troublesome slowness, and furthermore the chloroform solution itself, after a month or two at room temperature, became greenish and much weaker as shown by the lessening color response with both acid alcohol and the diazo reagent.[8] With a concentrated stock solution having 1 mg. for every 4 cc., and kept in the ice box, the loss of pigment within 2 months is negligible. It can be followed in the colorimeter by a comparison of the acid alcohol standard made from it with a mixture of 10 cc. of copper sulfate solution (10 gm. to 100 cc. of water) and 0.075 cc. of potassium bichromate solution (1 gm. in 100 cc. of water). Indeed, an inorganic standard of the sort described will probably prove best in the long run for routine colorimetric purposes. Slight alterations in the amount of bichromate suffice to turn the color toward the blue or green without essentially altering its value.

Control Observations.

The plan just outlined could be useful for our project only on the assumption that the bile from different liver regions has approximately the same pigment content per cubic centimeter. This cannot be taken as a foregone conclusion, if for no other reason than because the individual liver lobes receive portal blood from different visceral sources as a number of workers have shown.[9] To determine the actual case was our first step.

In six animals the neck of the gall bladder was tied off and the contents of the organ was removed by aspiration. A partitioning ligature was then placed upon the main duct as usual and, just above, a cannula connecting with a rubber balloon was inserted for collection of the bile that in later experiments was to flow instead to the bladder. The usual sample bag was then connected with the common duct and the laparotomy wound closed. Some of the animals were full fed at the time of operation, while others had fasted for 24 hours. All had access to food afterwards, but in general only those took it within the first 24 hours that had previously been denied. The factor was without notable influence on the result, as the table shows.

The liver regions supplying the two bags varied considerably from animal to animal because of differences in the duct arrangement. In two of the six instances more tissue was tributary to the sample bag than to the upper one.

[7] van der Bergh, A. A. H., and Snapper, J., *Deutsch. Arch. klin. Med.*, 1913, cx, 540.

[8] For a note on the changes in bilirubin kept in chloroform see Oppenheimer, C., in Fischer, G., Handbuch der Biochemie, Jena, 1909, i, 731.

[9] Bartlett, F. K., Corper, H. J., and Long, E. R., *Am. J. Physiol.*, 1914, xxxv, 36.

The table of results (Table I) has been arranged with reference to the question whether the bile in the sample bag gives any index to the pigment concentration of that elaborated by the rest of the liver.   It will be seen that this question is answered in the affirmative. The "bile strength," that is to say the pigment per cubic centimeter, is nearly the same for both portions of secretion, despite wide variations from animal to animal in the total fluid output and its bilirubin content (compare Experiments 2, 4, and 5).   This is a weighty point for it means that in the later work the bile strength can be used to gauge the concentration effected by the gall bladder.

The actual fluid in the upper bag and the calculated amount as determined from a knowledge of the quantity in the lower, or sample, bag and the relative pigment content of the two, assuming that there was an equal degree of concentration, differ but little, as would follow from the circumstance that the actual bile strengths of both proved to be nearly identical.   A calculation of the sort was regularly employed in the later work to gain an idea of how nearly the real amount of fluid reaching the gall bladder approximated that which should have reached it, judging from the partition of the hepatic tissue.

Some marked discrepancies will be noted between the actual fluid in the upper bag and the theoretical quantity as worked out from the partition of tissue.   The effect of the partitioning ligature could not always be exactly ascertained, as for example, when a single lobe possessed ducts draining to either side of it.   But this will not suffice to explain certain cases.   In Experiment 6 the rubber tube connecting said bag with the duct, being stiffer than ordinary, was at autopsy found sprung like a bow, with one end pressed upon the neighboring portal trunk and thus perhaps diverting blood to the region tributary to the lower bag with result in more active secretion there.   Whatever the cause of the other discrepancies these had no significance for our project.

The fact may be noted in passing that four animals out of five, with the instance of Experiment 6 excluded, yielded to the upper bag a bile that was relatively, if slightly, richer in pigment per cubic centimeter.   This was no accident, as will be shown further on.   There too, in Tables IV and V, are data that indirectly corroborate Table I.

Concentrating Power of the Emptied Gall Bladder.

Observations on the bladder were now begun. In a first series of experiments the organ was washed with salt solution, and left to fill with bile as it normally might fill, assuming that it empties on normal contraction.

A small slit was cut in the main bile duct at the point where the partitioning ligature was later to be laid on, and, through a silk catheter thrust up into the neck of the gall bladder, all bile was withdrawn and the organ washed with 0.9 per cent salt solution until the rinsings came away uncolored. Due care was taken to avoid overdistention, and the final emptying was accomplished by gentle pressure with moist sponges. The catheter was then withdrawn and ligatures were placed on the duct above and below the slit, to close it and divert the bile, part as usual into a bag, and the remainder to the empty bladder.

It may be asked whether the brief washing was effective; for a concentrated residuum of bile such as might be left clinging next the bladder mucosa would certainly complicate the findings. No difficulty was experienced on this score. The wall of the bladder is translucent, with the color of the contents shining through, and the efficacy of the washing can be controlled by direct inspection. Furthermore, the original bile was retained for comparison with that accumulating later in the sample bag. Practically always it was weak in pigment, relatively speaking,—whence one may conclude that any remnant of bladder bile left after the washing would act, if anything, to dilute in this respect the fluid entering later.

The contents at autopsy of the gall bladder was always so very viscid as well as dark that it was removed by rinsing with distilled water, and still further diluted prior to the withdrawal of a type portion. On opening the animal a clamp was laid on the bladder neck to prevent any passage of secretion from the hepatic duct. As a guide in the dilution, the assumption was made that as much bile had originally reached the gall bladder as was called for theoretically from the quantity in the sample bag and the proportion of hepatic tissue supplying the two; and the actual bladder contents was brought up to this amount. The mixture of bile and water was shaken repeatedly, allowed to stand several hours, and shaken again prior to the removal of 1 cc. for treatment with acid alcohol and comparison with a standard. The results with duplicate specimens showed that an even distribution of pigment had been brought about.

In all save one of this series of experiments, and in Experiment 6 of Table I, pigment values are expressed in "color units," the unit being the amount of pigment in 1 cc. of bile from the sample bag.

The period of experiment ranged in the five animals from $22\frac{1}{2}$ to 49 hours. To our great surprise the gall bladder even at the end

of the longest period held but a few cubic centimeters of bile, far less than the amount necessary for normal distention; while after 24 hours, the organ was practically collapsed in two out of three instances, yielding only 0.77 and 1.4 cc. of fluid, and in the third case was but half full. Could one suppose that the hepatic tissue tributary to the bladder had failed to secrete as usual? Or had its output undergone concentration to an extent commensurate with the findings? The latter proved to be the case. The dark, syrupy or tarry, bladder contents had from 3.18 to 10.8 times the pigment strength of the fluid in the sample bag, with an average of 7.1 (Table II). The contents of the tributary ducts was always by contrast thin and weak in pigment, like the bag bile. There were only a few drops to be had from the ducts, too little for colorimeter readings, so dilutions with water were compared directly with similar dilutions of bag and bladder biles. Sometimes they were in addition tested for cholates by Hay's method. The results confirmed the pigment findings.

The Concentrating Power of Full Gall Bladders.

With the gall bladder emptied as in these experiments its whole concentrating influence is brought to bear upon the secretion arriving little by little, from the liver. In this favorable circumstance lay not impossibly one cause of our results. To determine the real case a series of animals was studied, of which the bladders were filled with bile of known pigment content, before the wash catheter was withdrawn.

The dog bile to be introduced was collected in bags under aseptic conditions and kept on ice for periods up to 48 hours. In one instance, that of the material derived from the upper bag of Experiment 5, Table I, it proved to have been infected with a micrococcus of dubious pathogenicity, and this organism was recovered in pure culture from the gall bladder into which the bile was put (Experiment 1, Table III). Less concentration was effected by the bladder in this instance than in any other of the series.

To fill the bladder under a known pressure while preventing the escape of any fluid into the peritoneal cavity, a ligature was placed upon the duct containing the catheter just above the slit in its wall, and this was tied down as the catheter was withdrawn, thus becoming one of the partitioning ligatures. The catheter itself was connected with a sterile funnel containing the bile. The pressure of a column of bile 60 to 100 mm. high proved just sufficient to effect a

normal distention of the bladder. This is less than the pressure withstood by the sphincter of Oddi.[10] Needless to say, the tributary ducts shared the pressure conditions. In Experiment 7, a pressure such as develops upon duct obstruction[11] —300 mm.—was used.

Our expectation was to find at autopsy a marked stasis with dilatation of all the passages above the partitioning ligature, owing to secretion into them of more bile than the gall bladder could cope with. But the event was quite another. So rapidly was fluid withdrawn through the bladder wall that the increments of hepatic secretion proved insufficient in most instances to hold the organ distended. It was found nearly collapsed in Experiments 1, 4 and, 7, while in Experiment 6, in which alone a normal distention was observed, its capacity was unusually small, only 3.5 cc. The amount of bile introduced at operation and, to a less extent, that removed furnished for each case an approximate measure of capacity.

The inspissating activity of gall bladders left distended (Table III) proved to be little behind that of emptied ones (Table II). On the average the concentration of pigment was 6.4 times that of the bag samples, with a range from 3.6 to 8.9. The bile collected from the tributary ducts was thin with relatively little pigment, showing, as in the animals with emptied bladders, that the secretion had not been elaborated in condensed form. The greatest concentrations—to bile strengths, 8.1 and 8.9 times that of the bag samples— were effected during only 18 and 22 hours respectively. The amount of secretion acted upon in these instances as calculated from the quantities of bilirubin in bag and bladder and the fluid content of the former,—assuming both bile portions to have had the same pigment strength originally,—was for the first case 59.8 cc. which was reduced to 7.4 cc. by an organ of 9 cc. capacity, and for the second 26.6 cc. brought down to 3 cc. by a bladder holding at most 4.3 cc.

In Experiments 3 and 4, the bile introduced at operation had previously been concentrated 3.5 times—it was the upper bag contents of Experiment 2, Table V— and was syrupy with mucus. These changes did not prevent a further inspissation in the bladder to pigment strengths of 4 and 5.9 times respectively that of the bag samples.

[10] Judd, E. S., and Mann, F. C., *Surg., Gynec. and Obst.*, 1917, xxiv, 437.

[11] Herring, P. T., and Simpson, S., *Proc. Roy. Soc. London, Series B*, 1907, lxxix, 517.

In both Tables II and III it will be noted that the pigment found in the bladder frequently fell far short of the expected quantity (Table II, Experiments 4 and 5; Table III, Experiments 1, 3, 4, and 7). Must one suppose that part of the bilirubin reaching the organ had passed out through its wall, or was there diminished secretion above the partitioning ligature? Certainly pigment can pass the mucosa, for it has been observed histologically in transit;[12] but our observations upon the lymph indicate that the quantity thus removed is negligible. There is usually to be found coursing down the neck of the dog's gall bladder, and draining most of its extrahepatic portion, a large, turgid lymphatic yielding fluid in quantity when cut. We have frequently examined such fluid from gall bladders that held heavily pigmented bile. It was always practically colorless and failed to give positive reactions for bilirubin, though occasionally cholates were present, as shown by Hay's test. The direct passage of pigment into the blood stream cannot be ruled out, but it seems unlikely in view of these findings.

There remains the alternative of locally diminished secretion. The conditions in some cases would seem to have been highly favorable to this. The gall bladder in these instances was found at autopsy to be nearly collapsed, and so bound down by fresh adhesions between the adjacent liver lobes that its redistension could scarcely have been brought about by the normal secretory pressure. Under these cirsumstances, there may well have occurred a stasis in the tributary ducts at periods when secretion by the liver provided more fluid than the bladder could immediately concentrate. Direct proof of this was not obtainable because the necessary observations involved a severing of the very adhesions whereby the abnormal state was maintained. But it is interesting to note that the total output of bile pigment per kilo of animal averaged precisely the same (11.1 mg. in 24 hours, a normal amount) for the dogs of Tables II and III with a relatively small yield above the partitioning ligature, as for those of Tables I, IV, and V in which this was not the case. The fact suggests that whatever the cause of the small yield above the ligature, it resulted merely in a shift of the secretory activity with an unduly large output to the sample bag, and, by corollary, unwarranted expectations as to what should have been provided to the gall bladder.

The Bladder Utilized as a Duct.

The partitioning ligature of the preceding experiments could not be relaxed like the sphincter of Oddi. Fluid remained pent above it no matter how powerfully the gall bladder may have contracted. Here was an important departure from the normal; and the question arises whether the removal of fluid from the bile may not have been largely the consequence of pressure intermittently exerted by the bladder wall. For such reason another series of experiments was

[12] Aschoff, L., and Bacmeister, A., Die Cholelithiasis, Jena, 1909.

performed in which a partitioning ligature and sample bag were placed as usual, but the free tip of the bladder was connected by cannula with a second bag.

The tip of the bladder was seized with hemostats and a small slit made at the point where blood and lymph vessels were least abundant. The bile was removed by aspiration, and the usual flushing with salt solution; and a glass cannula with trumpet mouth and a least inside diameter of 2 to 3 mm. was fixed in place with a purse string suture. A rubber tube of the same bore, 4 to 8 cm. long, led from it to the rubber balloon. At autopsy this tube was clamped off as soon as the peritoneal cavity had been opened, to prevent the shifting of bile in either direction.

Four dogs were operated upon. The results are given in Table IV. The new cystic outlet was of somewhat larger caliber than the normal one, and at the most dependent portion of the bladder which was found practically empty at autopsy. The bile, urged by the secretory pressure and by gravity, had evidently run directly through, as through any other channel to the bag, being aided in two instances by a postoperative drawing together of the bladder wall which had much narrowed and shortened the organ. Nevertheless, the bile that had been submitted to it proved to be 2.3 to 4.8 times as concentrated as that in the corresponding sample bag. In view of such findings the results of Tables II and III cannot be attributed to the closed system existing above the partitioning ligature.

In the animals of Table IV the pigment content of the upper bag differed but slightly from the theoretical amount as calculated from the quantity in the sample bag and the proportion of tissue tributary to each. In this respect the findings were nearer perfection than in the control series of Table I. The absence of any cause for local portal obstruction such as was provided by the upper cannula in the dogs of Table I may have been responsible for this.

In final illustration of the concentrating activity, three experiments originally intended as controls will be reported, which were carried out prior to realization of the bladder capabilities. To obtain the bile from above the partitioning ligature in these instances, a cannula of large bore was thrust through a cut in the bladder wall into the neck of the organ and secured there. The duct from the right side of the right central lobe enters so high up that there is often no true cystic duct, and in order to avoid obstruction of this

tributary, the cannula was not pushed down to its level but left with a tiny pouch of bladder mucosa about its mouth. That the influence of the pouch was far from negligible is shown by the results. The upper bag yielded a bile syrupy with mucus and considerably richer in pigment per cubic centimeter than the thin, sample fluid (Table V).

It may be recalled that in the controls of Table I the bile from the upper bags was generally slightly the more concentrated. Now it so happens that in the dog the gall bladder wall extends some distance down the duct, the macroscopic character of the latter being often first evident below the entrance of the highest branch from the liver. Others have noted this before us. Indeed, both in the dog and in man a new gall bladder may develop out of the remnant of bladder mucosa left by a cholecystectomy that has failed to include the cystic duct.[13] It follows that a ligature placed on the neck of the bladder to block the organ off, as in the experiments of Table I, may frequently fail in some part of its function. To such a happening do we attribute the slightly greater concentration of most of the upper biles of Table I. For, as Table IV demonstrates, a transient exposure of the bile to but a fraction of the bladder wall results in a reduction of its bulk.

Influences of the Ducts.

For the purposes of the present study the duct system proper has been deemed without influence upon the fluid it conveys. But in view of the great activity of the gall bladder, is such an assumption warranted? For practical purposes it is, as we shall show in an accompanying paper. The ducts instead of withdrawing fluid from the bile tend to dilute it slightly with a watery product of their own.

Peculiar Character of the Bile Acted upon.

No such deeply pigmented biles as the gall bladder yielded at the end of our experiments are found under normal conditions. Normal bladder bile of the dog is often light yellow, and, at most, of a

[13] Rost, F., *Mitt. Grenzgeb. Med. u. Chir.*, 1913, xxvi, 710. Haberer, H., and Clairmont, P., *Verhandl. deutsch. Ges. Chir.*, 1904, xxxiii, pt. 2, 81.

medium brown tint, whereas that now referred to was always dark, and frequently brown-black. It may be recalled that the activities of the bladder had been brought to bear on but a fraction, and sometimes a small fraction, of the total secretion. But this is not the sum of the matter. For quantitation of the bag samples showed clearly that the liver had furnished an abnormal secretion, one extremely rich in pigment and small of bulk.

The bile of healthy dogs has ordinarily from ¼ to ½ mg. of bilirubin in every cc., and practically never as much as 1 mg.;[14] whereas that of our animals contained after operation more than 2 mg. per cc. usually, only once less than 1 mg., and in one instance 6.8 mg. Such plenitude was attained almost wholly at the expense of the fluid output, as shown both by direct measurement of the latter and by the fact that the total pigment elaborated by the liver in the 24 hours immediately after operation was, if anything, only a little increased over the normal. The day to day output of bile varies greatly, a fact that Stadelmann[15] has emphasized. He gives figures for two dogs weighing 16 to 17 kilos which show that they secreted about 288 cc. of bile per 24 hours, that is to say slightly more than ⅔ cc. per kilo in 1 hour. But the six dogs of our Table I yielded respectively $\frac{1}{18}$, $\frac{1}{17}$, ⅓, ⅓, $\frac{1}{50}$, and ⅓ cc. per postoperative hour, amounts that are with one exception greatly below Stadelmann's average. The more indirect data of the other tables confirm the point thus illustrated. According to Stadelmann and Hooper and Whipple,[5] the normal bilirubin output is about 1 mg. per pound of dog in 6 hours, or for present purposes 8.8 mg. per kilo of animal in 24 hours. In our animals the pigment put forth after operation ranged from 8 to 13.4 mg. per kilo in 24 hours in uncomplicated cases, with an average of 11.1 mg. The two complicated cases (Experiments 7 of Table III and 5 of Table II) that were left from this computation yielded 7.3 and 15.4 mg. respectively.

One cause for the peculiar character of the postoperative bile at once suggests itself. Fasting animals, as is well known, yield but little bile, and this heavily pigmented and with a high percentage of solids.

In a dog followed by Stadelmann the secretion of the first 24 hours after food was withdrawn had a bulk only half that in the period immediately previous and in a second 24 hours less than one-third. The total pigment output, though,

---

[14] The pigment studies of Hooper and Whipple with fistula animals may be consulted upon this point.

[15] Stadelmann, E., Der Icterus und seiner verschiedenen Formen, Stuttgart, 1891.

underwent no change. The observation will explain many, perhaps all, of our instances. The food intake of our dogs and their water intake as well, in the preliminary and experimental periods together, was far below the normal. Nearly all of the animals that took food in the 24 hours following operation had fasted through a like period prior to it.

Nature of the Concentrating Faculty.

It would be highly interesting to know the exact composition of the scanty, dark, postoperative liver bile. For the withdrawal of fluid from it in the gall bladder is accomplished entirely through osmosis and diffusion, and the concentration thus achieved will necessarily vary with the bile character.

Brand[2] has found that both the hepatic and bladder biles have the Δ of the blood; and through comparative analyses he obtained clear evidence that the concentration effected in the bladder comes about at the expense of the inorganic salts which are removed as what is, practically speaking, a normal saline solution. Hammarsten[3] and others confirm this, in that they too find a less quantity of inorganic salts in bladder bile than in liver bile, but correspondingly more of substances having large molecules.

The fact that the limit of biliary concentration is the Δ of the blood will explain our observation that the gall bladder contents showed no greater degree of inspissation after 48 hours (Experiments 3 and 4, Table II) than after one-third to one-half this period (Experiments 1 and 2, Table II, and all of Table III). The shifting of constituents whereby a reduction of the bile volume comes about takes place so rapidly (Table IV) that it must be practically complete within a few hours. In the lack of comprehensive analyses, the suitability of the heavy, postoperative liver bile of our experiments for concentration in the gall bladder cannot be profitably discussed. But there is every reason to suppose that its limit of concentration would be reached far sooner than that of the normal secretion, which is, by contrast, watery.

Under the operative conditions we have employed, some obstruction to the passage of fluid away from the bladder must not infrequently have been caused. The large lymphatics derived from the viscus course close beside the main duct in the gastrohepatic omentum, and the slight dissection required to place the partitioning

ligature, together with the pull of the latter, cannot but have some-times compromised such delicate vessels. As offsetting this in that it favored resorption of fluid may be put the preliminary cleansing of the gall bladder mucosa with salt solution. But in the course of many observations upon normal dogs we have only rarely encoun-tered a mucous layer next the bladder wall. Usually the organ holds a thin, practically homogeneous syrup, and the mucosa is clean. That a moderate mucus admixture need not greatly hinder concen-tration is shown by Experiments 3 and 4 of Table III with a secretion already rendered syrupy and more than thrice concentrated by the gall bladder of another animal. Taken as a whole, the conditions of our experiments were probably rather unfavorable to the concen-tration of bile.

Bladder Fistulæ.

Practically all quantitative studies of the bile from day to day have been carried out on fistula animals, with the gall bladder as a link in the fistulous system. The bile runs from the ligated common duct through the gall bladder and out by a slit in its tip which is sewn fast to an opening in the abdominal wall. Our findings of Table IV show that this practice involves great possibilities of error as regards actual bulk of the liver secretion, since it may be much reduced in transit. Perhaps, though, the bladder wall soon loses its concentrating faculty, owing to pathological change. Instances have been described of temporary obstruction to a fistulous outlet in which the bladder was found filled, not with inspissated bile, but with hydropic fluid,[16] the product of a damaged mucosa.[12] Never-theless, the possibility of concentration by the bladder must be kept in mind in reviewing the data of fistula experiments.

Functions of the Gall Bladder.

There appears to be little general realization of the physiological uses of the healthy gall bladder which has now become a favorite surgical trophy. Yet several attested purposes the organ has.

[16] Kölliker, A., and Müller, H., *Verhandl. physik.-med. Ges. Würzburg*, 1856, vi, 435.

Pawlow's assistants[17] have shown that an intermittent discharge of bile takes place into the duodenum during the passage of chyme from the stomach, but ceases with this. Thereafter, until food is again taken, only a very occasional spurt of secretion passes the sphincter of Oddi—about once an hour in the dog.[13] The first bile expelled into the chyme is recognizably bladder bile, being syrupy and usually darker than that coming later, though both escape under pressure, in small spurts or jets, at short intervals. After cholecystectomy a great difference is observed. Bile dribbles continuously from the ampulla of Vater[13] and during fasting may fill the duodenum and be voided as such in the stools.[18] The disturbance of function thus indicated is not without a bad effect on the digestive processes,[18] masked though this usually is; and Rost has described a striking anatomical change that is common in man and the dog after cholecystectomy; *viz.*, a general dilatation of the bile passages. His finding has been confirmed by numerous observers. The dilatation does not occur when the sphincter of Oddi is destroyed.[10] Rost applies the term "biliary incontinence" to the continuous escape of secretion into the intestine after removal of the gall bladder. The incontinence is associated with an abnormal relaxation of the sphincter[10] which latter, however, frequently recovers its tone as duct dilatation ensues.[13]

Such activities as are more or less directly illustrated by these facts fall into three categories. The gall bladder acts like a distensible bag interpolated into a rigid system of tubes, to minimize extremes of pressure when bile comes rapidly or in large quantity from the liver and its escape into the intestine is prevented by the sphincter. The bag in question is rendered capacious not so much through its size as by a singular ability to reduce the bulk of the fluid reaching it. Small wonder that after cholecystectomy the ducts dilate and the sphincter gives way![19] The organ is also propulsive, delivering bile to the duodenum when needed. But such service is subsidiary, if essential, to the storage of bile. During those periods when the duodenum is empty the bladder husbands the bile for future use, and through its concentrating activity is enabled to retain very nearly all of the liver output when the interval from one gastric digestion to another is not unduly long. An illustration of the point may be given:

[17] Bruno, G. G., Dissertation, St. Petersburg, 1898, and Klodnizki, Dissertation, St. Petersburg, 1898; cited by Babkin, B. P., Die äussere Sekretion der Verdauungsdrüsen, Berlin, 1914, 344. Bruno, G. G., *Arch. Sc. biol. St. Pétersbourg*, 1899, vii, 87.

[18] Hohlweg, H., *Deutsch. Arch. klin. Med.*, 1912, cviii, 255.

[19] Judd, E. S., *Ann. Surg.*, 1918, lxvii, 473.

The period of most abundant bile formation in a normal animal coincides roughly with that during which chyme leaves the stomach. Secretion has diminished markedly 10 or 12 hours after the ingestion of food,[15] and thereafter continues slowly to lessen. A normal dog of 9 kilos fed every 12 hours, that is to say under favorable conditions for secretion, will form, in every 12, about 90 cc. of bile.[20] Much less will be put out when a feeding is omitted. Now if such a dog be supposed to have a gall bladder holding 10 cc., which is well below the average capacity,[21] and the organ be endowed with the ability to concentrate the bile sixfold, which in view of our experimental findings is not too much to assume, there should be room in it for 60 cc. of liver bile. That so much will actually come from the liver is doubtful. The hourly small spurt into the duodenum during a fast is not indicative of a tensely filled gall bladder but may be directed to the maintenance of sterility of the passages.

In all likelihood the gall bladder has functions in addition to those outlined. Its importance as a reservoir is perhaps less in animals that, like man, eat frequently, than in species such as the dog that habitually go long periods without food. There may be reasons for the concentrating activity besides the reduction in fluid bulk. And the need for mucus in the bile is unexplained. The elaboration of mucus in quantity is, like the concentrating activity, a function of the bladder as distinct from the ducts. Indeed, the receptaculum chyli has been much too often considered a mere diverticulum in the duct system. The special character of its influence upon the bile deserves emphasis as demonstrating the highly purposeful differentiation of the organ. The fact that few ills follow upon removal of the normal gall bladder means merely that the body has adapted itself to the loss, not that the loss is unimportant. In this connection the surgeon would do well to remember that uncertainty as to function and confidence in readjustment are at best questionable motives for adventures in ablation.

[20] To judge from Stadelmann's instances.

[21] According to Mann (Mann, F. C., *New Orleans Med. and Surg. J.*, 1918, lxxi, 80), the average capacity for a dog of 8 kilos is 16.6 cc. The individual variation is great, as our Table III shows. Mann's figure indicates that we had to do with unusually small bladders, a view supported by our more recent experience.

SUMMARY.

The bile coming at one time from different portions of the liver of the dog has nearly the same amount of pigment per cubic centimeter. With this determined we have studied the power of the gall bladder to concentrate bile directed to it, using as criterion the pigment strength of a sample collected throughout the period of experiment from a duct branch. The extent and rapidity of the concentration are alike remarkable. A gall bladder emptied at the beginning of one experiment and left to fill from the liver, concentrated the 49.8 cc. of bile reaching it in $22\frac{1}{2}$ hours to 4.6 cc., that is to say reduced its bulk 10.8 times; while another bladder left distended with a bile of known constitution and receiving in addition fresh increments from the liver concentrated the secretion 8.9 times in 22 hours. A series of five emptied bladders concentrated the bile coming to them in about 24 hours on the average 7.1 times, or a little more than the 6.4 times of seven organs left full. The conditions in both cases were relatively unfavorable to the withdrawal of fluid from the bile because this takes place by osmosis and diffusion, with the ultimate Δ always that of the blood, and the secretion in our animals was notably rich in solids as an indirect result of the operation.

The rapidity with which fluid is withdrawn through the wall of the bladder may be judged from some experiments in which a bag was connected with the tip of the organ by a large cannula. Merely in its passage through the bladder the bile was concentrated 2.3 to 4.8 times. The finding indicates a potential source of error in observations upon samples of bile obtained from fistulous channels of which the bladder forms a part.

The bile ducts do not withdraw fluid from the secretion they convey but tend to dilute it, as we shall show in a companion paper. The restriction of the concentrating activity to the receptaculum chyli is good evidence that the latter has special significance for the organism. The nature of this significance is briefly discussed.

TABLE I.

Control Instances.

	Period.	Bag.	Lobes drained.	Weight of tissue drained.	Bile amount. As calculated from Tissue.	Pigment output.	Actual.	Pigment. Calculated total.	Actual total.	Amount per cc.	Bile strength.	Remarks.
	hrs.			*gm.*	*cc.*	*cc.*	*cc.*	*mg.*	*mg.*	*mg.*		
Experiment 1; ♂; 12¾ kilos (fasting).	24	*Sample.*	Right lateral and caudate.	154.0			7.2		48.9	6.8	1	About 74 gm. of tissue in right side of right central lobe totally obstructed by the ligature; partition of tissue only approximately known.
		Upper.	Left central and lateral; left papillary; left side of right central.	244.0	11.4	9.5	9.9	77.5	64.5	6.5	0.96	
Experiment 2; ♀; 8 kilos (fasting).	24	*Sample.*	Right lateral and caudate.	91.5			7.6		25.0	3.3	1	Very early pregnancy.
		Upper.	Remainder of liver.	161.0	13.4	12.9	10.3	44.0	42.4	4.1	1.25	
Experiment 3; ♀; 6½ kilos (full fed).	24	*Sample.*	Left lateral and central.	105.0			27.3		48.1	1.76	1	
		Upper.	Remainder of liver.	130.7	34.0	26.8	23.3	60.0	47.2	2.0	1.15	

Experiment	No.	Portion	Tissue								Remarks
Experiment 4; ♀; 7 kilos (full fed).	24	Sample.	Left lateral, half of left central; caudate and right lateral.	138.0	19.1	24.4	20.4	28.0	30.0	1.1 — 1	Partition of tissue only approximately known; very early pregnancy.
		Upper.	Remainder of liver.	94.0				19.0	26.1	1.4 — 1.28	
Experiment 5; ♀; 9 kilos (some food).	17	Sample.	Right lateral, caudate, and two-thirds of left lateral.	154.0	95.5	76.8	95.5	70.0	70.0	1	Partition of tissue only approximately known; pigment in units, not mg.; bile of upper bag infected.
		Upper.	Remainder of liver.	210.0				68.5	76.8	1.12	
Experiment 6; ♂; 8 kilos (fasting).	24	Sample.	Right and left lateral and caudate.	149.0	33.8	15.5	33.8	48.0	48.0	1	Pigment in units; probable interference with portal flow to tissue drained by upper bag; both biles syrupy.
		Upper.	Remainder of liver.	105.0				19.0	15.5	0.82	

Some of the animals had received no food in the 24 hours prior to operation. The fact finds parenthetical record in the first column of the table. All of the animals had access to food afterwards, but in general only those ate that had fasted previously.

TABLE II.

Emptied Gall Bladders.

Experiment	Period	Bile	Lobes drained	Weight of tissue drained	Kind of bile	Bile amount. As calculated from — Tissue	— Pigment output	Actual.	Pigment. Calculated total.	Actual total (units).	Amount per cc.	Bile strength.	Remarks.
	hrs.			gm.		cc.	cc.	cc.	mg.		mg.		
Experiment 1; ♂; 8 kilos.	22½	Bag.	Right lateral and caudate.	77.0	Golden brown, thin.			19.1		19.1		1	Hay's test yields similar findings; gall bladder half collapsed; right pneumothorax.
		Bladder.	Remainder of liver.	202.0	Dark brown, syrupy.	50.2	49.8	4.6		49.8		10.8	
Experiment 2; ♀; 9½ kilos.	24	Bag.	Right and left lateral; left central; caudate.	198.0	Dark brown, thin.			15.0		15.0		1	Hay's test yields similar findings; gall bladder collapsed.
		Bladder.	Right central; papillary.	90.0	Brown-black, tarry.	6.8	6.65	0.77		6.65		8.6	
Experiment 3; ♂; 11½ kilos.	46½	Bag.	Caudate; right and left lateral and left central.	296.0	Dark brown, thin.			47.5		47.5		1	Gall bladder half collapsed.
		Bladder.	Right central; papillary.	127.0	Brown-black, tarry.	20.6	22.8	4.9		22.8		4.65	

Experiment 4; ♂; 11 kilos.	Bag.	Left central and lateral lobes.	141.0	Dark brown, thin.	107.0		77.0		77.0			1	Gall bladder half collapsed.
	Bladder.	Right lateral and central; papillary; caudate.	196.0	Brown-black, tarry.		43.8	5.4		43.8			8.1	
Experiment 5; ♀; 4½ kilos.	Bag.	Right and left lateral; left central; caudate.	95.0	Dark brown, thin.			27.75		59.8	2.15		1	Probable stasis in ducts leading to gall bladder owing to adhesions which latter bind down; pigment in mg.
	Bladder.	Right central; papillary.	43.5	Dark brown, syrupy.	12.7	4.45	1.4	27.4	9.6	6.86		3.18	

Filled Gall Bladders.

Experiment	Period. hrs.	Bile.	Lobes drained.	Weight of tissue drained. gm.	Kind of bile.	Bile amount — As calculated from — Tissue. cc.	Bile amount — As calculated from — Pigment output. cc.	Bile amount — Actual. cc.	Pigment — Calculated total. mg.	Pigment — Actual total. mg.	Pigment — Amount per cc. mg.	Bile strength.	Remarks.
Experiment 1; ♂; 7 kilos.	24	Bag.	Left lateral and central; right lateral; caudate.	173.0	Dark green, thin.			31.0		76.7	2.48	1	At autopsy gall bladder bound down by fresh adhesions; 10.5 cc. of infected bile containing 10.3 mg. of pigment had been left in it at 70 mm. pressure.
		Bladder.	Right central and papillary.	69.0	Brownish black, semifluid.	(12.4)* / 22.7	(1.6) / 11.9	3.3	(30.6) / 40.9	(3.9) / 14.2		3.6	
Experiment 2; ♀; 7 kilos.	18	Bag.	Right lateral and caudate.	67.0	Medium brown, thin.			25.5		14.3	0.56	1	9 cc. of bile containing 20.1 mg. of pigment left in gall bladder at 70 mm. pressure.
		Bladder.	Remainder of liver.	195.0	Dark brown, viscid.	(74.2) / 83.2	(50.8) / 59.8	7.4	(41.6) / 61.7	(28.5) / 48.6		8.1	
Experiment 3; ♀; 9 kilos.	24½	Bag.	Right lateral and caudate.	95.0	Dark brown, thin.			25.0		64.5	2.6	1	11 cc. of syrupy bile containing 25 mg. of pigment left in gall bladder at 100 mm. pressure.
		Bladder.	Remainder of liver.	256.0	Dark brown, viscid.	(67.4) / 78.4	(8.9) / 19.9	5.25	(174.0) / 199.0	(23.0) / 48.0		4.0	

Experiment	No.		Part of liver	Wt.	Character of bile							Remarks
Experiment 4; ♀; 8¾ kilos.	25	Bag.	Right and left lateral and caudate.	188.0	Dark brown, thin.	(29.5) 41.3	37.75	(53.0) 79.7	(33.0) 67.7	1.8	1	
		Bladder.	Remainder of liver.	147.0	Very dark brown, viscid.	(13.4) 25.2	4.3		(24.1) 50.8	1.75	5.9	11.8 cc. of syrupy bile containing 26.7 mg. of pigment left in gall bladder at 65 mm. pressure.
Experiment 5; ♀; 6 kilos.	22	Bag.	Right lateral and caudate.	105.0	Dark brown, thin.	(35.2) 39.5	20.6	(61.7) 67.1	36.0		1	
		Bladder.	Remainder of liver.	180.0	Very dark brown, viscid.	(22.3) 26.6	3.0		(39.0) 44.4	1.75	8.9	4.3 cc. of bile containing 5.4 mg. of pigment left in gall bladder at 70 mm. pressure.
Experiment 6; ♀; 6½ kilos.	24½	Bag.	Right lateral and caudate.	59.0	Dark brown, thin.	(18.8) 22.3	8.3	(61.6) 68.7	27.1	3.3	1	
		Bladder.	Remainder of liver.	134.0	Brownish black, thick.	(20.0) 23.5	3.5		(65.4) 72.5		6.7	3.5 cc. of bile containing 7.1 mg. of pigment left in gall bladder at 90 mm. pressure.
Experiment 7; ♂; 7½ kilos.	24	Bag.	Right lateral and caudate only?	98.5	Greenish brown, thin.	(55.5) 60.0	33.5	(79.4) 87.3	47.9	1.4	1	
		Bladder.	Remainder of liver.	163.0	Dark brown, viscid.	(5.9) 10.4	1.4		(8.4) 16.3		7.4	At autopsy gall bladder bound down by fresh adhesions; 4.5 cc. of bile, containing 7.9 mg. of pigment had been left in it at 300 mm. pressure.

* The figures in brackets = contribution of the liver to the gall bladder contents; those unbracketed = sum of this and of material introduced at time of operation.

TABLE IV.

Bladder Fistula.

	Period.	Bag.	Lobes drained.	Weight of tissue drained.	Kind of bile.	Bile amount. As calculated from Tissue.	Bile amount. As calculated from Pigment output.	Bile amount. Actual.	Pigment. Calculated total.	Pigment. Actual total.	Pigment. Amount per cc.	Bile strength.	Remarks.
	hrs.			*gm.*		*cc.*	*cc.*	*cc.*	*mg.*	*mg.*	*mg.*		
Experiment 1; ♂; 12 kilos.	24	*Control.*	Right lateral and caudate.	98.0	Thin, medium brown.			17.5		42.1	2.4	1	Gall bladder flaccid, contains 0.3 cc. of bile; cannula from it 3 mm. in least diameter.
		Bladder.	Remainder of liver.	249.0	Syrupy, dark brown.	44.4	49.5	16.2	106.9	119.0	7.4	3.1	
Experiment 2; ♂; 9¾ kilos.	24¾	*Control.*	Right lateral and caudate.	118.0	Thin, medium brown.			10.75		23.6	2.2	1	Gall bladder shrunken, contains 1.4 cc. of bile pressed back into it at autopsy; cannula from it 3 mm. in least diameter.
		Bladder.	Remainder of liver.	255.0	Thin, dark brown.	23.2	25.7	11.2	51.0	56.3	5.0	2.3	

Experiment													Partition of tissue
Experiment 3; ♂; 15¾ kilos.	9	Control.	Caudate and part of right lateral.	64.0	Thin, medium brown.			10.3		22.6	2.2	1	Partition of tissue approximate; gall bladder contains 0.6 cc. of deeply stained mucus; lower bag contents infected with an organism that does not alter the pigment; cannula 2.5 mm. in least diameter.
		Bladder.	Remainder of liver.	315.0	Ropy, brownish black.	50.7	47.4	9.8	111.1	103.9	10.6	4.8	
Experiment 4; ♂; 11¾ kilos.	23¼	Control.	Caudate and right lateral.	73.0	Thin, dark brown.			15.0		35.1	2.3	1	Gall bladder shrunken, empty; cannula 3 mm. in least diameter.
		Bladder.	Remainder of liver.	205.5	Syrupy, much darker brown.	42.3	41.1	10.25	98.8	96.1	9.4	4.1	

TABLE V.

Cannula in Bladder Neck.

	Period.	Bag.	Lobes drained.	Weight of tissue drained.	Kind of bile.	Bile amount. As calculated from Tissue.	Bile amount. As calculated from Pigment output.	Bile amount. Actual.	Pigment. Calculated total.	Pigment. Actual total.	Pigment. Amount per cc.	Bile strength.	Remarks.
	hrs.			*gm.*		*cc.*	*cc.*	*cc.*	*mg.*	*mg.*	*mg.*		
Experiment 1; ♂; 11¾ kilos (full fed).	20	*Lower.*	Right lateral and caudate.	200.0	Medium brown, thin.			43.0		43.0		1	Pigment in units, not mg.
		Upper.	Remainder of liver.	305.0	Similar color (thick?).	65.6	56.5	40.0	65.6	56.5	0.66	1.41	
Experiment 2; ♂; 8 kilos (fasting).	21¾	*Lower.*	Upper part of right lateral lobe.	43.0	Dark brown, thin.			5.2		3.4		1	Partition of tissue only approximately known. Stasis in ducts to upper bag?
		Upper.	Remainder of liver.	316.0	Dark brown syrupy.	38.2	100.0	28.8	25.0	65.6	2.3	3.48	
Experiment 3; ♀; 10¼ kilos (fasting).	26	*Lower.*	Right lateral and caudate.	115.0	Dark brown, thin.			12.5		42.3	3.4	1	
		Upper.	Remainder of liver.	245.0	Dark brown, syrupy.	21.3	33.5	20.0	90.0	113.6	5.7	1.68	

General Description of the Tables.

The most important data given relate to the relative pigment concentrations, or bile strengths, of the portions of secretion derived from the different liver regions of the same animal, that of the so called sample bag being taken as a standard. Table I shows that when the influence of the gall bladder is ruled out, the portions differ little in pigment value. The other tables present evidence that this value undergoes a manifold increase in bile submitted to the bladder.

Since the pigment strength is much the same for the unmodified secretion from different portions of a single liver, it becomes possible to ascertain approximately the quantity of bile reaching a gall bladder during a given period from the pigment accumulation in it as compared with that in a type specimen of the liver bile collected into a bag. Thus, for example, in Experiment 1 of Table II the bladder contained 49.8 units of pigment (a unit = pigment in 1 cc. of sample bile) in only 4.6 cc. of fluid, as compared with 19.1 units in the 19.1 cc. of the type specimen. It follows that 49.8 cc. of secretion had reached the bladder. Data obtained in this way have been given place in the tables. So too have figures on bile output and pigment quantity derived from a knowledge of the contents of the sample bag and the proportion of tissue tributary to it and to the bladder. In Experiment 1 of Table II the output of 77 gm. of liver, as collected in the sample bag, amounted to 19.1 cc., while 202 gm. supplied the bladder. It follows that the latter should have received 50.2 cc. of bile, an amount closely approximating the real one (49.8 cc.) as calculated out on the basis of actual pigment content. For the purposes of a comparison with this last, the total pigment that should theoretically have reached the bladder has been calculated out on the basis of the tissue partition and the pigment in the sample bag.

In the experiments of Table III the use of a foreign bile to distend the bladder has complicated the expression of results. To determine the amount of secretion and of pigment coming from the liver during the experiment, it was necessary to deduct from the ultimate findings the amounts introduced. This has been done. The figures in brackets represent the amounts of bile and of pigment furnished by the liver, and the unbracketed figures just beneath represent the sum of such quantities and of those introduced. Thus in Experiment 1, 10.3 mg. of pigment was put in the gall bladder and 14.2 mg. found at the close of the experiment. The liver then had contributed 3.9 mg. of pigment, or 1.6 cc. of fluid, judging from the pigment strength of that in the sample bag. Since 10.5 cc. of fluid had originally been introduced, the total acted upon by the gall bladder was 11.9 cc., and in the reduction of this to 3.3 cc. a 3.6 fold concentration was effected.

PHYSIOLOGICAL CAUSES FOR THE VARIED CHARACTER OF STASIS BILE.

By PEYTON ROUS, M.D., and PHILIP D. McMASTER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATE 4.

The fluid encountered at operation in the obstructed bile passages of human beings is of notably various character. Even in cases free of infection all gradations may be found between a black, tarry material and the watery, colorless "white bile" that has long puzzled surgeons. The causes for this diversity are not immediately evident in clinical instances because of the numerous complicating factors which give to each an almost individual peculiarity. One looks in vain for a clue to them in such a book as that of Kehr[1] which describes in detail the findings in many hundred operations upon diseased bile passages. But they are readily ascertained through experiment. The different, and in general opposed, functions of the gall bladder and ducts are principally responsible—infection aside—for the protean character of stasis bile.

Method.

Dogs have been mostly employed for the work, with some cats and *rhesus* monkeys. Many animals used for concurrent observations on other themes were available. Obstruction to the bile ducts was produced by tying and cutting, with the excision, where possible, of a piece, and at difficult points by ligatures laid on in series. In dogs and cats the danger of a restoration of continuity by cutting through of the silk thread was found to be negligible when that used was of large caliber. The operations were performed aseptically under ether anesthesia, and the abdominal wound was closed in three layers. Infection and other complications were rare. Save where specifically mentioned, instances showing them have been ruled from consideration. After some days or weeks

---

[1] Kehr, H., Drei Jahre Gallensteinchirurgie. Bericht über 312 Laparotomien am Gallensystem aus den Jahren 1904–06, Munich, 1908.

the animals were chloroformed and the ducts, their contents, the liver, and the hepatic vessels were carefully studied. Bits of the tissue and drops of the stasis bile were placed on agar and in bouillon, and the hepatic tissue was examined histologically.

The common duct of the dog and cat is formed from three or more main hepatic branches, high up into one of which the gall bladder empties. The arrangement varies greatly from animal to animal, and by taking advantage of special instances one may obstruct every large duct in turn, now in connection with the gall bladder and again separately, in association with a total obstruction, or with a local one so small as to be insignificant for the organism. In this way it is possible to test whether any duct has functions peculiar to itself. There is, properly speaking, no cystic duct in the dog or cat, owing to the entrance just below the bladder neck of a tributary from the right side of the right central lobe, but in the monkey there is a slender one, which, like that of man, empties into the common duct, an arrangement which much limits the obstructive permutations possible to the experimenter.

When the material was sufficient, quantitative estimations were made of the pigment and cholates in the stasis bile. In our opinion the results so obtained are not to be accepted without reservation because bilirubin, at least, undergoes changes on incubation in the gall bladder;[2] but they sufficeto indicate the trend of affairs. Hooper and Whipple's[3] modification of the Salkowski test was used to estimate pigment. The method has been discussed at some length in a preceding paper.[4] For bile salts, the Foster and Hooper[5] amino nitrogen method was employed when the material was sufficient, and Hay's sulfur test when it was not. The amino-acid determinations were carried out for us in Dr. Van Slyke's laboratory. The Pettenkofer test for bile salts could not be used because it yields a positive result with cholesterol. To disclose the presence of the latter the Liebermann-Burchard method was employed.

Contrasting Types of Stasis Bile.

Whenever an obstructed bile duct was left in communication with the gall bladder the stasis bile later found proved to be heavily pigmented, and syrupy, ropy, or even tarry, according to whether the period of obstruction had been short or long.

Experiment 1.—In sixteen dogs, two cats, and one monkey obstruction of the common duct was produced, or of one or more of its hepatic tributaries in such wise that the gall bladder still communicated with the channels in stasis.

---

[2] Hammarsten, O., Lehrbuch der physiologischen Chemie, Wiesbaden, 8th edition, 1914.

[3] Hooper, C. W., and Whipple, G. H., *Am. J. Physiol.*, 1916, xl, 332.

[4] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1921, xxxiv, 47.

[5] Foster, M. G., and Hooper, C. W., *J. Biol. Chem.*, 1919, xxxviii, 355.

The changes that took place in the stasis bile were the same, whether total obstruction had been produced or not. In animals killed after 2 to 4 days the gall bladder and the ligated ducts connecting with it were tensely, though not greatly, distended with a syrupy, dark brown bile. 2 or 3 days later the color of the fluid was noted to be definitely green-brown, and after 10 days to 2 weeks of stasis it was a green-black, and the contents of the gall bladder was mucinous or even tarry, while that of the connecting ducts was a heavy syrup. In the next 3 or 4 weeks the bladder bile underwent no important change but the fluid in the ducts gradually thickened to a jelly which gave Hay's reaction for bile salts in high dilution. Further our observations did not go.

The contents of ducts separately ligated and of the obstructed common duct blocked off from the gall bladder was entirely different.

Experiment 2.—In nineteen dogs, three cats, and four monkeys, the common duct was obstructed and the gall bladder neck as well, or else one or more large hepatic ducts separately tied and cut.

The stasis fluid in these instances was at first brown, then green but definitely less pigmented, and finally, after 10 days or more of stasis, clear, often completely colorless, even in jaundiced animals, or at most of a pale yellow, and usually without sufficient cholates to give Hay's or Udranszky's test. That from the cat was syrupy and in one case a tenacious jelly, but the yield of the dog and monkey was watery with a slight, translucent, glairy admixture. No greater contrast to the inspissated biles of Experiment 1 could have been devised. Yet both types of stasis fluid were frequently obtained at one time from the same animal (Figs. 1 and 2).

Experiment 3.—Obstructions were so placed on the bile channels of twenty-one dogs, three cats, and two monkeys that some of the large ducts in stasis were deprived of their connection with the gall bladder, while others still possessed it.

At autopsy both sets of ducts were equally distended, the one with a heavy, green bile giving Hay's test in high dilution, the other with the colorless or lightly tinted fluid above described (Fig. 1). In the contents of the finest duct ramifications visible to the eye on section of the liver tissue, like differences were discernible. Several apparent exceptions in which a green bile was found where a colorless fluid had been expected served to emphasize the invariable nature of the rule.

On search, a communication with the gall bladder was discovered in every such instance, either by way of a fistula or through reestablishment of the old connection.

Causes for the Differing Types.

It is clearly evident how the contents of a green system, as we may call one connected with the gall bladder during stasis because of the characteristic hue, comes to be highly pigmented and at last tarry. In a companion paper we have demonstrated that the normal gall bladder effects a great and rapid concentration of the bile. One has only to suppose that the organ still functions in some measure during stasis to explain the heaping up of pigment in it and the connecting ducts. The change from brown to green is, for the most part at least, a simple oxidation of bilirubin to biliverdin.[2] The thickening to a heavy syrup and eventually to a tar or jelly occurs through the gradual accumulation of a mucinous nucleoprotein which is a normal product of the bladder mucosa.[2]

What, now, is the derivation of the colorless material distending a "white system," one obstructed out of connection with the gall bladder? There are several possibilities, but a discussion of them is unnecessary since our findings point in a single direction. The thin, colorless fluid is not bile at all but a product of the duct wall that has gradually replaced the small amount of hepatic secretion originally pent up.

Experiment 4.—A greater or less portion of the common duct with, in some instances, the trunks of the larger hepatic ducts was isolated in five dogs by ligating and cutting it above and below. After various periods up to 12 days the animals were chloroformed and examined. The isolated duct segment was found uninflamed but greatly distended in every case—up to a diameter of about 1 cm. on the average—and held several cubic centimeters of colorless and watery, or thinly mucinous, fluid, identical in its obvious characters with that of a white system of ducts as above described. All of the animals had become jaundiced as result of the total obstruction, yet none of the duct fluids was bile-stained and the two that were submitted to Hay's test gave a negative response. Cultures attested their sterility.

Experiment 5.—In a female dog of 11.5 kilos a segment of common duct was isolated and connected by means of a glass cannula and flexible rubber tube with a small, empty rubber balloon, which was left in the abdominal cavity when the abdominal wound was closed. Recovery from the operation was prompt, and the

animal remained in excellent condition during the 6 days prior to reexamination. The bag now held 8.5 cc. of a clear, sterile, watery fluid, slightly alkaline to litmus, and with a specific gravity of 1.011. The mucosa of the duct was not inflamed. A few degenerating epithelial cells separated from the fluid on standing, and when looked at in long column the latter had a faintly greenish cast, doubtless as result of changes in the bile pigment originally left in the duct. Hay's test for cholates was negative. The dog had not yet developed a tissue icterus.

These experiments demonstrate that the mucosa of the duct secretes a fluid of its own and is not prevented from so doing by a pressure sufficient to stretch considerably the tough and rather inelastic duct wall. The degree of distension observed was about equal to that in the closed ducts above the isolated segment. In this relation it is interesting to remember that the pressure within the ducts rises during stasis in the dog to equal that of a column of bile approximately 300 mm. high.[6,7] The amount of fluid produced in Experiment 5 in the absence of such a pressure obstacle was no negligible one—8.5 cc. in 6 days from a strip of mucosa about 2 cm. long and 0.7 to 0.8 cm. broad when the duct was laid open longitudinally.

Histological Changes.

The hepatic tissue in connection with obstructed ducts filled with colorless bile does not differ in the least in appearance, even after weeks of stasis, from that giving into a green system in the same animal. When the ducts from part of the liver have been left open there will be noted a general dilatation of the blocked channels, a slowly developing, orderly, interlobular cirrhosis, a few pigment thrombi between the parenchymal cords, and a more or less marked parenchymal atrophy in the region of stasis with compensatory hypertrophy elsewhere. When obstruction is total and jaundice has been present for some weeks one will find in addition marked parenchymal icterus and many intralobular bile thrombi, but still no differences referable to the green and white systems. Coursing beside the distended colorless ducts of the latter, and away from the liver, may be seen lymphatics turgid with

[6] Herring, P. T., and Simpson, S., *Proc. Roy. Soc. London, Series B*, 1907, lxxix, 517.

[7] Mitchell, W. T., Jr., and Stifel, R. E., *Bull. Johns Hopkins Hosp.*, 1916, xxvii, 78.

fluid colored a bright yellow with bilirubin, and yielding a positive Hay's reaction. It is clear that the liver must be forming bile which is somehow prevented from entering the usual channels. The preventive agent can scarcely be other than the colorless secretion of the duct walls which has gradually backed up within them.

Development of a White System.

A systematic study has been made by us of the various steps in the development of a white system in the dog. During the first few days of obstruction the duct contents is still pigmented, appearing indeed somewhat more so than normal bile, owing to the conversion of part or all of its bilirubin into the much darker biliverdin. After about a week, as a rule, the pigment has become much less in amount, though in the lack of any criterion as to its original quantity, which varies greatly, of course, from animal to animal, examples cannot be given.

By the 10th or 11th day the duct contains a practically colorless fluid. In eleven dogs with total obstruction lasting from 8 to 27 days and icterus in varying degree, it was completely colorless, and devoid of cholates. So too in three cats with pronounced jaundice after 11 to 14 days of obstruction. Many instances could be cited of perfect white systems in animals with a partial obstruction, but these have less interest, owing to the fact that the occurrence of icterus was prevented in their case through a vicarious elimination of bile by the unobstructed liver portions. We have said that very few formed elements are present in the duct fluid. Cholesterol is practically absent as shown by the Liebermann-Burchard test which is occasionally negative and at most weakly positive. All these facts are as true for the contents of branches from single lobes, separately obstructed, as for that of the large channels.

Authorities are not agreed as to the precise point of escape of the bile from obstructed ducts but it is known to be close to the margin of the lobuli. According to Heidenhain[8] and most workers it is situate at the junction of the intralobular bile capillaries with the collecting channels of Glisson's capsule. Bürker[9] places it within the lobuli but

[8] Heidenhain, R., Studien des physiologischen Instituts zu Breslau, Leipsic, 1868, No. 4, 234.

[9] Bürker, K., *Arch. ges. Physiol.*, 1900–01, lxxxiii, 241.

near to their periphery. However this may be, one can suppose that in the gradual formation of a white system the fluid that accumulates in the ducts, diluting and replacing their original content, escapes at the same point as the bile. Certainly it must escape far back toward the lobuli, else the ducts in Glisson's capsule would be observed to contain bile, not colorless fluid when the liver is cut open. One may vision a slow upward current of duct secretion meeting and opposing a more rapid one downwards of bile in the region of the duct radicles, and the escape of both together through the walls.

In the monkey a "white bile" completely devoid of pigment and cholates is not obtainable, owing to the great distensibility of the ducts, so that relatively large amounts of stasis bile collect in them; to the short period during which they remain obstructed when cut between ligatures; and finally to one of several factors now to be discussed, which, acting likewise in the dog, often prevent the elaboration of a perfect "white bile." These are: (1) derivation of the white system from an hepatic lobe having another outlet which is patent; (2) the presence of gall bladder mucosa in the white system; (3) long continued obstruction.

In another connection[10] we have furnished evidence that the bile radicles which unite to form each primary hepatic duct fail in the dog to anastomose in any significant degree with those of other ducts. Each drains what may be termed a separate watershed, and when its outlet is blocked, as when the duct is tied, the tributary region suffers to its outermost limits, as is shown by the eventual sharp line of demarcation between normal liver parenchyma and that of the region in stasis, a line which follows closely the anatomical limit of the obstructed ducts. But physiologically the separation is not quite absolute, as the present work shows. Repeatedly in the course of it we have had opportunity to note the influence of a free duct on the contents of a white system, having its ramifications in the same tissue mass. Under such circumstances the fluid of the white system is regularly yellow with bilirubin and contains cholates, although other white systems deriving from entire lobes of the same liver have contents colorless and negative for bile salts. The fact that the pigment

---

[10] McMaster, P. D., and Rous, P., *J. Exp. Med.*, 1921, xxxiii, 731.

in the affected white system is bilirubin, not biliverdin as in old stasis biles, argues its recent production. The most reasonable explanation of its presence would seem to be a constant slight secretion of bile into the ducts as result of a lowering of pressure in them consequent in turn upon a slight continual leakage from the white system over into the adjoining unobstructed region. How and where this leakage occurs cannot be said. In the monkey there is evidence for a considerable amount of it in the development regularly of a broad zone of transition between the liver tissue that is atrophic as the result of transmitted pressure from obstructed ducts and the adjoining normal parenchyma.

We have called attention in another paper to a difficulty encountered in attempts to block off completely the gall bladder of the dog without encroachment on the duct. The bladder mucosa often extends below the neck of the organ, to or beyond the entrance of the first channels from the liver, as is readily seen when the duct is laid open. The development of a new gall bladder after cholecystectomy is probably traceable to this arrangement, for it never occurs when the segment of duct in question immediately next the bladder has been ablated.[11] So great is the concentrating activity of the bladder upon the bile that when a ligature is laid on the neck of the organ the small residual portion left below may suffice, as we have shown, to effect a considerable reduction in the fluid bulk of the secretion.[4] It is to such a reduction in fluid bulk, whereby a little bile is enabled to enter a white system, that some of our anomalous instances should be attributed. In these cases when the duct distended with pale yellow bile was slit open a still more distended segment of characteristically thin walled bladder mucosa disclosed itself below the ligature which had been supposed to exclude it entirely. The neighboring ducts which had been separately obstructed held by contrast a colorless fluid.

Finally, in several dogs in which a white system and a general jaundice had existed for 6 weeks or more the stasis fluid was yellow and contained cholates in the absence of the factors just mentioned. The duct wall was now stretched very thin. Lessened secretion from it, or seepage through it, may have occurred.

[11] Haberer, H., and Clairmont, P., *Verhandl. deutsch. Ges. Chir.*, 1904, xxxiii, pt. 2, 81.

The Changes in a Green System.

The activities of the gall bladder during stasis merit special attention because they perhaps have no small share in the production of gall stones. The changes that occur within a white system are all in the direction of dilution and replacement of the bile. Those in a green system on the other hand seem at first view to be wholly the result of progressive biliary concentration. Within the first few days the bladder contents turns greenish black and as weeks elapse it thickens to a tar or jelly, while similar though less marked alterations occur in the fluid of the connecting ducts. It has required quantitative observations to show that here is no gradual accumulation of pigment; and in a preliminary note we have expressed this erroneous view.[12] What really happens is a progressive conversion of bilirubin to the greatly darker biliverdin, whereby a gradual disappearance of much of the pigment is masked. The point can be readily demonstrated by diluting out the bladder bile with water. The bile obtained after a week or 10 days of obstruction, though colored a green-black, dilutes out through the yellow-brown of bilirubin, to a bright yellow which only disappears in relatively large quantities of water. After another week the green persists to a higher dilution, but the proportion of water required to bring about a total disappearance of color is not so great. Finally after 5 weeks or more in the dog, and much sooner in the monkey, the yellow tint is missing when water is added to the bile and only a clear green is got which fails to survive much dilution. The disappearance of pigment thus indicated is also shown by titrations carried out according to Hooper and Whipple's method. At the latest period of stasis that we have studied—44 days, in the dog—the bladder contents still reacts characteristically with acid alcohol, and colorimetric readings against a bilirubin standard can be made. The amount of pigment found per cubic centimeter is now not more than twice that of many normal biles, instead of six to ten times the quantity as during the first 10 days.

How soon after the production of a green system does the concentrating activity of the gall bladder cease, and what are the maximum alterations effected by it? These questions are not readily answered.

[12] Rous, P., and McMaster, P. D., *Proc. Soc. Exp. Biol. and Med.*, 1920, xvii, 143.

TABLE I.

Character of Stasis Bile from the Gall Bladder.

Dog No.	Duration of obstruction.	Contributing liver lobes.	Liver per cent.	Jaundice.	Character of stasis fluid.	Diluting to.	Pigment per cc.	Sodium taurocholate per cc.	Hay's test + at dilution of.	Remarks.
	days						*mg.*	*mg.*		
1	2	Right and left central; papillary.	43	0	Dark brown, syrupy.	Yellow.	2.7	32.2	1 in 80	Other lobes unobstructed.
2	4	All except caudate and papillary.	83	Slight.	Brown-black, syrupy.	"	5.9	35.0	1 " 64	Other lobes unobstructed. No tissue jaundice but bilirubin in blood and urine.
3	9	Caudate, right lateral; half of right central.	42	0	Thick olive-black, syrupy.	"	2.8	36.7	1 " 64	Other lobes unobstructed.
4	9	All except caudate.	88	0	Thick olive-black, syrupy.	Greenish yellow.	4.3	34.0	1 " 40	Other lobes unobstructed.
5	9	Right central; papillary.	32	+	Viscid, green-black.	"	3.2		1 " 20	Other lobes obstructed.
6	9	Main liver mass.	73	+	Viscid, green-black.	"	5.7		1 " 40	Other lobes obstructed.
7	10	Right and left central; papillary.	43	0	Thick greenish black syrup.	"	3.8	10.2	1 " 64	All other lobes except caudate obstructed.
8	12	Right and left central.	38	+	Thick greenish black syrup.	Yellow-green.	3.0		1 " 32	Other lobes obstructed.

9	34	Right central and papillary.	31	0	Thick green-black syrup.	Green.	1.9	63.1	1 in 192	Caudate and right lateral also obstructed.
10	36	Right half of right central.	12	0	Tarry, dark green.	Yellow.	1.0	16.4	1 " 128	Other lobes save caudate and right lateral are also obstructed.
11	36	Right central and papillary.	26	0	Tarry, dark green.	Green.	0.9	56.6	1 " 64	Other lobes excepting right lateral and a part of caudate are obstructed.
12	44	Main liver mass and papillary.	65	0	Tarry, green-black.	Yellowish green.	1.2	8.5	1 " 256	Part of caudate lobe also obstructed.

The inspissatory changes come about entirely through osmosis and diffusion, and are limited by the Δ of the blood.[13]   After the first few days the bladder and ducts undergo no further dilatation, their capacity when they are taken together being now only twice to four times the normal. It follows that the stasis bile will be mostly a derivative of that purveyed by the liver during the first few days of obstruction.   And since the constitution of ordinary bile in substances of large molecule varies much, it follows that the small space available for its accumulation may in some individuals come to be occupied largely by substances of one sort to which the bladder wall is impermeable, and again by those of another.   This fact is reflected in the irregularities of Table I.

In none of the fluids recovered after 2 days or more of stasis was so great a concentration of pigment found as in some bladder biles acted upon by the normal organ for 24 hours.[4]   After 9 days of stasis the pigment is still unincreased, and later there is a gradual diminution in it.   One may conclude that the maximum pigment concentration is effected during the first day or two of obstruction, and that thereafter the activity of the gall bladder in this regard practically ceases.

Quantitative observations on cholesterol were not made because the bladder mucosa itself is recognized to be a source of the substance. Cholates though were estimated in two ways, as already stated,—by Foster and Hooper's amino-acid method and by Hay's sulfur test carried out on progressive dilutions of the bile.

Both of these methods as used in connection with stasis bile are open to criticism.   An inspissated bile long kept at body temperature may very well come to contain other substances besides salts of taurocholic acid that fail to come down in boiling alcohol and will yield amino-acids on hydrolysis.   Derivatives of the abundant nucleoprotein in special might well do this.   Hay's sulfur test is not only given positively by any substance that lessens surface tension, but the lessening ordinarily caused by cholates may be hindered by other bile constituents. Thus, for example, we have several times found that a watery solution of sodium taurocholate which yielded a positive Hay's test when mixed with a given amount of water, failed to do so in the presence of a very slight trace of bile although the latter itself contained cholate but not in quantity sufficient to elicit a reaction in the dilution employed.   There are then several possible explanations for the differences in result of the two methods (Table I).

---

[13] Brand, J., *Arch. ges. Physiol.*, 1902, xc, 491.

The general indications obtained by both methods are that bile salts, unlike pigment, may increase in concentration as stasis progresses.

The gradual viscid thickening is the result of the persistent elaboration by the bladder wall of a mucinous material identified by Hammarsten as a nucleoprotein.[2] This is not produced in any quantity by the ducts. At first only the contents of the receptaculum chyli are thickened with it, but later it extends into the larger and smaller passages, and finally the most minute ones visible to the unaided eye are distended with tiny plugs of green jelly. In this connection it is noteworthy that the distribution of pigment and cholates throughout the green system eventually becomes an approximately even one. At first these are principally massed in the gall bladder, as would follow from the localization of the concentrating activity to this latter.

Experiment 6.—In thirteen dogs, one cat, and one monkey, with green stasis systems of 2 to 36 days duration a clamp was placed upon one of the tributary ducts at autopsy and the fluid obtained from above was compared with the bladder contents as regards pigment and cholates. So little material was yielded by the duct that Hay's test of graduated watery dilutions, and a direct color comparison with similar dilutions of the bladder contents, were the best methods available. They regularly indicated at first marked differences between the two biles, which tended to disappear later. Thus after 4, 9, and 10 days of obstruction, respectively, in three dogs the pigment was only 54, 25, and 30 per cent as strong in the duct fluid as in the gall bladder, whereas in three other dogs kept for 34, 36, and 36 days, its relative strength was 80, 86, and 100 per cent.

More factors influence the changes in a green stasis system than in a white one. The differing permeability of the bladder wall for different substances, and the eventual clogging of the mucosa which will vary in its effects with the substance; the continued production of mucus; the activity of the ducts to dilute the bile; these and doubtless other moments interact to alter the stasis fluid. The eventual tendency is toward replacement of the bile with duct secretion and mucinous material from the bladder.

The Pressure in Green and White Systems.

The fluid in obstructed bile ducts is pent under a day-to-day pressure averaging about 300 mm. of bile in the dog,[7] a sufficient evidence that there are considerable barriers to its escape. These barriers would appear to be equally effective for the green and white systems. The distension of the ducts is the same in both when present in a single liver, and, what is perhaps more significant, the hepatic regions pertaining to the two show no noteworthy differences. We have pointed out in a previous paper[10] that the pressure in regions of stasis has effect in a diversion of the portal stream with local parenchymal atrophy and a compensatory hypertrophy elsewhere. The changes may be readily followed in the dog liver, where they are marked after but a few weeks. Now were the duct pressure in a green or a white system much greater than in its fellow there should be a reflection of this in differing degrees of parenchymal change. Such are not found. The activity of the gall bladder, while sufficient to determine the character of the stasis bile, is, then, without enduring influence on the stasis pressure. The fact that the pressure developing when ducts are suddenly obstructed is the same as that on their continued stasis[4,7] suggests that the prime moment in its maintenance is the secretory activity of the liver.

Some Results of Infection.

Consideration has been given thus far only to happenings in the absence of infection. The entrance of this latter may bring with it innumerable complexities. Two that have come under our eye will be put on record, since they throw light upon the problem of obstruction in man.

The fluid elaborated by infected ducts is sometimes no longer thin but so viscid as practically to occlude the ducts. In a dog from which we were making day-to-day collections of the bile from different portions of the liver, infection occurred of one of the duct systems drained by rubber tubes. Almost at once its output became thickly mucinous and practically lost the biliary character, being but faintly tinted with bilirubin whereas the companion bile that served as control was still thin and dark. At autopsy the liver

proved to be normal; and portions of the control bile when incubated with organisms from the infected ducts failed to thicken. There is little doubt that here we had to do with cholangitis rendered obstructive by a thick secretion from the duct walls; that is to say, just such a condition of affairs as is supposed to be operative in the catarrhal jaundice of human beings.

Infection may so change the gall bladder that a white system develops where a green is expected. In one of our dogs chronic infection led to a thickening of the bladder wall, and this organ and all of the obstructed ducts connected with it were found distended with a colorless, syrupy fluid that failed to give Hay's reaction but contained small ropes of old pus and numerous micrococci. There is good reason to suppose that in this instance the infection prevented an inspissation of bile by the bladder.

DISCUSSION.

It has long been taken for granted that the walls of the ducts influence the bile as do those of the gall bladder, and this view has colored the interpretation of pathological findings. That it is incorrect the present observations show. The activities of ducts and bladder are opposite in nature, but of such different magnitude that those of the bladder determine the picture when the bile is submitted to both. It is a curious fact that the duct walls, though intimately related to the tissue forming the bile, themselves secrete a fluid that is colorless even when the animal is heavily jaundiced. The glands of the wall behave in this respect like those forming the tears and saliva and those of the gastric mucous membrane,[14] as contrasted with the sweat glands and kidneys which put out bilirubin during icterus. It is true that the fluid in a white system with walls stretched thin after long standing total obstruction may be lightly tinted with bilirubin and contain cholates; but these substances not improbably come from the liver under such circumstances. The fact may be recalled in this relation that mucous surfaces in general become permeable to bile pigment when inflamed.[14]

[14] Krehl, L., Pathologische Physiologie, Leipsic, 9th edition, 1918.

"White biles" have been obtained from human beings with extreme fatty degeneration of the liver[15] as well as after duct obstruction. The instances on record leave little doubt that a liver with injured parenchyma sometimes yields a colorless fluid having scarcely any resemblance to true bile. Our present findings warrant the suggestion that the fluid is derived from the duct walls, while the liver cells proper have practically ceased secreting. However this may be, there is no doubt that the ducts are the source of "white bile" in obstructive instances. In them the integrity of the parenchyma is attested by the resumption of secretion into the old channels when the impediment has been removed.[16] The condition of affairs while the obstruction holds is essentially similar to that studied by Heidenhain[8] in his classic experiments on the introduction under pressure of sodium indigotate into the ducts. The strange fluid, whether indigotate or white bile, fills all the extralobular passages, preventing any bile from entering them, and its surplus escapes with the bile itself through the walls of the duct radicles at or near the margins of the liver lobuli. When pressure is relieved the bile once more takes its way into the proper channels, flushing out ahead of it the strange fluid. Secretion by the liver is never inhibited, only diverted, a fact abundantly proven by the jaundice that ensues when the obstruction has been total.

In many instances in man of white bile from obstructive causes the hepatic duct or a branch of it has alone been blocked, but in others the common duct is occluded and the gall bladder and all the passages fill with a colorless fluid.[16] Here is an apparent contradiction to our rule on the origin of green and white systems. But a green system can only be produced when the gall bladder is capable of concentrating bile during stasis. Lacking this ability the organ becomes a mere diverticulum in a white system. And it is with such incapable gall bladders that one has to do in these anomalous instances. Aschoff and Bacmeister[17] have emphasized the fact in

---

[15] Ritter, E., *Compt. rend. Acad.*, 1872, lxxiv, 813. Robin, A., *Compt. rend. Soc. biol.*, 1884, xxxvi, 115.

[16] Kausch, W., *Mitt. Grenzgeb. Med. u. Chir.*, 1911, xxiii, 138. Fischler, F., Physiologie und Pathologie der Leber, nach ihrem heutigen Stande mit einem Anhang über das Urobilin, Berlin, 1916.

[17] Aschoff, L., and Bacmeister, A., Die Cholelithiasis, Jena, 1909.

connection with hydrops that the normal gall bladder does not distend with fluid when tied off at the neck but draws gradually down into a small, thick walled globe containing a little mucous jelly. This happens even during the jaundice of total obstruction, as we have had occasion to note. Hydrops, as Aschoff and Bacmeister rightly say, is the expression of a change in the bladder wall such that fluid is elaborated by it instead of withdrawn through it. When an organ pathologically active in this way, or one merely indifferent to the bile, stands in connection with an obstructed duct system there will inevitably occur a gradual replacement of the original stasis bile with a secretion derived from the duct walls. We have furnished a specific instance in the animal already mentioned that was operated upon for the production of a green system, but in which a white developed instead, in connection with a bladder thick walled from chronic infection. Kölliker and Müller[18] noted as far back as 1856 in a dog with an ill cared for gall bladder fistula that a glairy, colorless fluid collected when the fistulous opening closed for a day or two, which was replaced by bile after some hours of drainage.

A recognition of the physiological influences which make for the production of green and white stasis systems should bring some order into the chaos of observations on stasis bile. One is enabled to say with certainty that here the concentrating activity of the gall bladder is mainly responsible for the character of the fluid found, and that there it is the product of the ducts and in certain instances of an hydropic bladder. But the diverse infections to which the biliary passages are liable bring with them innumerable complexities. One we have recorded in the sudden mucinous thickening of duct bile, with obstruction as a result.

The differing activities of the bladder and ducts bear directly upon the problem of cholelithiasis. Our observations leave little ground for surprise over the fact that stones of the hepatic duct or its branches are relatively infrequent and give but little trouble clinically. A greater or less degree of obstruction of these channels must often occur: it is inevitable to inflammatory or neoplastic changes in the liver tissue. But such local stasis as may thus be caused is followed,

[18] Kölliker, A., and Müller, H., *Verhandl. physik.-med. Ges. Würzburg*, 1856, vi, 435.

as the present experiments show, not by a concentration of the stagnating bile, but by its dilution and replacement with a fluid from which one of the principal substances forming stones, bilirubin, is often completely absent and the other, cholesterol, sometimes practically so. The fluid is thin, like the liver bile itself, and both will readily find a way around stones that only partially occlude a duct.

The participation of the gall bladder completely changes the conditions and the ultimate prospect. We have shown how rapidly the organ acts to inspissate the bile, even in the absence of stasis.[4] Aschoff and Bacmeister bring evidence that most stones have their beginning in an uninfected and approximately normal gall bladder. In their view cholesterol falls out of the incubated bile in crystalline form and deposition takes place thereon; while Naunyn[19] and many others believe that bacteria and cellular debris constitute the nuclei of formation. Whatever the true case, this much at least is certain, that the enlargement of stones is by a deposition out of solution or suspension. The activity of the gall bladder to concentrate the bile cannot but be of profound importance in this connection. Indeed, if one accept the cholesterol hypothesis of stone origin it assumes primary significance. The very rapid "growth" of stones that have passed into the common duct and cause partial obstruction, as described by Naunyn, and the secondary formation in such cases of stones just above, are alike attributable in large part to the influence of the bladder upon repeated fresh increments of bile. And the danger of total obstruction under such circumstances is rendered greater by the thickening of the bile with bladder mucus.

We have already pointed out that the upper portion of the cystic duct often has the physical characters of the bladder wall and with them its concentrating ability.[4] This latter fact will go far to explain the development of stones in the duct after cholecystectomy, an occurrence not infrequent in the days before duct ablation was practised.

Aschoff has an aphorism to the effect that the essential and common cause of all gall stones is biliary stasis. One might say for many

[19] Naunyn, B., A treatise on cholelithiasis, Sydenham Society translation, London, 1896.

cases, as coming nearer to the actual event, that it is biliary inspissation.

Stones rarely develop as a result of continuous obstruction. Under such circumstances, as we have shown, bile soon ceases to come from the liver into the closed passages, and that which at first assembles becomes thickened with mucus from the bladder and gradually diluted with the secretion of the duct walls. In none of our old bladder biles was any particulate matter found except desquamated cells.

How do these facts bear on the present vogue of cholecystectomy? It is evident from them that the concentrating activity of the gall bladder renders it a menace during intermittent stasis and whenever the bile itself is of such kind that stones readily form out of it. The more normal the organ, or to speak precisely, the more of the concentrating faculty it retains, the greater is the danger. On the other hand, the surgeon should realize that the removal of a normal gall bladder entails, as we have pointed out in a companion paper,[4] functional disturbances that are none the less significant because the body adjusts itself to them. Furthermore, as Oddi originally showed, cholecystectomy is often followed by a marked and permanent dilatation of all the ducts so that they come to hold much bile. The accumulated secretion is separated from the intestine only by a weakened sphincter, and ascending infection of the ducts has been noted to occur.

In patients with a tendency to stone formation it would seem wise to prevent, so far as possible, concentration of bile by the gall bladder. A simple expedient suggests itself to this end, namely frequent feeding. During fasting periods not only is bile stored in quantity and concentrated by the bladder but the secretion as it comes from the liver is rich in solids.[20] The passage of chyme through the duodenum is accompanied by an expulsion of the bladder contents[21]

[20] Stadelmann, E., Der Icterus und seiner verschiedenen Formen, Stuttgart, 1891.

[21] Bruno, G. G., Dissertation, St. Petersburg, 1898, and Klodnizki, Dissertation, St. Petersburg, 1898; cited by Babkin, B. P., Die äussere Sekretion der Verdauungsdrüsen, Berlin, 1914, 344. Bruno, G. G., Arch. sc. biol. St. Pétersbourg, 1899, vii, 87.

and its replacement with an abundant, thin flow from the liver. Viewed as prophylaxis, the oftener this can happen the better.

SUMMARY.

The gall bladder and ducts exert opposite influences upon the bile. The ducts fail to concentrate and thicken it with mucus as the bladder does, but dilute it slightly with a thin secretion of their own that is colorless and devoid of cholates even when the organism is heavily jaundiced. The fluid may readily be collected into a rubber bag connected with an isolated duct segment. It continues to be formed against a considerable pressure, and, in the dog, is slightly alkaline to litmus, clear, almost watery, practically devoid of cholesterol, and of low specific gravity to judge from the one specimen tested. In obstructed ducts separated from the gall bladder, or connecting with one so changed pathologically that the concentrating faculty has been lost, such fluid gradually replaces the small amount of bile originally pent up. It is the so called "white bile" of surgeons.

When obstructed ducts connect with an approximately normal gall bladder the stasis fluid is entirely different, owing to the bladder activity. At first there accumulates in quantity a true bile much inspissated by loss of fluid through the bladder wall, darkened by a change in the pigment, and progressively thickened with bladder mucus. As time passes duct secretion mingles with the tarry accumulation and very gradually replaces it. The inspissation of the bile, as indicated by the pigment content, is at its greatest after only a day or two of stasis.

The differing influences of the ducts and bladder upon the bile must obviously have much to do with the site of origin of calculi and their clinical consequences. The concentrating activity of the bladder cannot but be a potent element in the formation of stones. We have discussed these matters at some length. Intermittent biliary stasis is admittedly the principal predisposing cause of cholelithiasis; and the stasis is to be thought of as effective, in many instances at least, through the excessive biliary inspissation for which it gives opportunity. In this way a normal gall bladder can become,

merely through functional activity, a menace to the organism. In patients with the tendency to stones frequent feedings may lessen the danger of their formation.

EXPLANATION OF PLATE 4.

FIG. 1. White and green stasis systems after 11 days of total obstruction with jaundice. Retouched photograph. The gall bladder and connecting ducts, *A*, *A*, *A*, are dark with heavily pigmented green bile, while the branches, *B*, *B*, of a separately ligated duct distended with "white bile" are translucent and practically colorless.

FIG. 2. "White bile" and green from the same animals. The contrasting specimens, *A* and *B*, were obtained after 22 days of obstruction in a dog with one large duct left open, and consequently no icterus; and *A'* and *B'*, after 26 days of total obstruction with icterus.

FIG. 1.
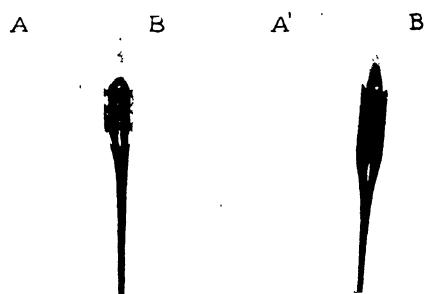
FIG. 2.

STUDIES ON THE D'HÉRELLE PHENOMENON.*

By ANDRÉ GRATIA, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 5 AND 6.

INTRODUCTION.

About 3 years ago d'Hérelle[1] found that stools of patients recovering from bacillary dysentery contain a filterable substance which is able to dissolve cultures of the Shiga bacillus and that a few drops of the dissolved culture reproduces the same phenomenon upon addition to another culture, and so on, indefinitely. Through these different passages the lytic property, instead of decreasing by dilution, is on the contrary increased and retains its activity even after several years.

Such a continuous transmission of the lytic property occurs only if the transfer is made into living cultures of the Shiga bacillus. Hence d'Hérelle concluded that the lytic agent is a filterable virus parasitic on the Shiga bacillus. To this supposed virus he has given the name of bacteriophage. He discovered similar bacteriophages for *B. coli, B. typhosus, B. paratyphosus* A and B, and some other bacilli.

Salimbeni[2] claimed to have isolated the bacteriophage, which he described as a myxameba possessing spores so minute that they are capable of passing porcelain filters.

Kabéshima,[3] on the other hand, denies the living nature of the bacteriophage and considers it merely as a catalyzer secreted by the leucocytes of the infected intestine and capable of activating a lytic proferment present in the body of the microbes.

The observations of Bordet and Ciuca[4] also make very doubtful the parasitic nature of the bacteriophage. These authors injected guinea pigs intraperitoneally

---

* Most of the findings here reported have already been presented as preliminary notes in *Compt. rend. Soc. biol.*, 1921, lxxxiv, 275, 750, 751, 753, 755.

[1] d'Hérelle, F., *Compt. rend. Acad.*, 1917, clxv, 373; 1918, clxvii, 970; 1919, clxviii, 631; *Compt. rend. Soc. biol.*, 1918, lxxxi, 1160; 1920, lxxxiii, 52, 97, 247.

[2] Salimbeni, *Compt. rend. Soc. biol.*, 1920, lxxxiii, 1545.

[3] Kabéshima, T., *Compt. rend. Soc. biol.*, 1920, lxxxiii, 219, 471.

[4] Bordet, J., and Ciuca, M., *Compt. rend. Soc. biol.*, 1920, lxxxiii, 1293, 1296.

three times at 5 day intervals with cultures of *B. coli*. 1 day after the last injection the peritoneal leucocytic exudate exhibited the properties of the bacteriophage of d'Hérelle; namely, a continuous transmission of lytic action.

Bordet and Ciuca also observed that a culture of *B. coli*, once dissolved by an immunized exudate and then filtered, is able either to dissolve a second culture of *B. coli* or to inhibit its growth in broth. But neither this dissolution nor the inhibition is absolute, since a few organisms always resist the dissolution and multiply, although slowly. The latter bacilli are distinguished from the original culture by certain characteristics: they resist the lytic agent but have now themselves acquired the lytic property and become lysogenic, or capable of inducing dissolution in a culture of normal *B. coli*. Moreover, when planted on slanted agar a mucoid, sticky culture results; also they are less phagocytable and more virulent for guinea pigs than the normal culture from which they were derived. All these properties are preserved even after passage through animals.

Thus there seems to arise under the influence of the lytic substance a race of bacilli which is adapted to this substance and is characterized by new and transmissible properties such as the increased virulence. Bordet and Ciuca call this race *"modified B. coli,"* and they infer that under the influence of the peritoneal exudate mentioned a variation of the colon bacilli occurs in the sense that they now secrete an autolysin which dissolves their own cells, with the exception of a few resistant organisms which survive and continue to produce the lytic secretion. Hence Bordet and Ciuca conceive the phenomenon to be that of a transmissible microbic autolytic property.   .

The facts as outlined are of fundamental importance, since they relate not only to the problem of the lysis itself but also to such disputed questions as the appearance of new races, the heredity of acquired characteristics, and the nature of virulence.

Influence of Hydrogen Ion Concentration.

We first established the influence of the hydrogen ion concentration on the lytic action[5] which, as is known, manifests itself in two ways, either in inhibiting the growth of *Bacillus coli* in freshly planted broth cultures or in dissolving a culture already grown. As the former phenomenon is more easily observed than the latter, the inhibiting action of the lytic agent will be chosen as a criterion in the following experiments.

Experiment 1.—Two tubes of plain broth were seeded with the same small quantity of *B. coli*; *i.e.*, with 1 drop of a 24 hour broth culture. Immediately

---

[5] We are indebted to Dr. Bordet for a strain of *B. coli* with which he carried on his studies, together with a quantity of the corresponding lytic agent. The experiments were conducted with this material.

afterward 10 drops of the lytic agent were added to the second tube. In this condition, it is known that the first tube grows normally while the second remains perfectly clear for a certain period, which can be easily measured.

We have repeated this experiment with broths of varying hydrogen ion concentrations and observed that the inhibition is markedly influenced by the reaction of the medium; it is faint in a slightly acid (pH 6.8), neutral (pH 7), or even slightly alkaline broth (pH 7.4), but much stronger in a more alkaline broth (pH 8 or 8.5).

In a slightly acid broth (pH 6.8) containing 10 drops of lytic agent, *Bacillus coli* grows after 2 to 3 hours almost as well as in normal broth. But this early growth disappears very quickly by dissolution. After a few hours, however, a renewed growth begins and this time develops normally, reaching its maximum after 24 to 36 hours. Then another dissolution occurs, but this process is slow and incomplete. On the following day the culture becomes somewhat more opaque again.

Hence the impression arises of a succession of waves of growth and redissolution, at each wave of growth *Bacillus coli* becoming more resistant.

On the other hand, in a highly alkaline broth (pH 8.5) the inhibition is much more striking and it is only after 36 to 48 hours that growth appears.

Between these extremes we have observed all intermediate degrees, the inhibition increasing with the increase of the alkalinity.

Lysis and Microbic Variation.

Bordet and Ciuca have clearly demonstrated that the lytic agent spread on the surface of a young culture of *Bacillus coli* on slanted agar clarifies the culture by dissolution, but that after a certain period of incubation one can detect a few irregular colonies which have the distinctive characteristics of the resistant *Bacillus coli* (Fig. 1, *A*). We observed a very similar picture by merely allowing the normal culture of *Bacillus coli* to age. An old agar slant of *Bacillus coli* shows a uniformly dull film on which appear very distinctly, here and there, small vitreous colonies. The first impression is that of a contamination, but these colonies are undoubtedly *Bacillus coli* and their distribution reproduces that of the resistant colonies

observed by Bordet and Ciuca (Fig. 1, *B*).[6] On transplanting the material of the desiccated film between the vitreous colonies, no growth occurs. The organisms originally in this material are now dead. On the other hand, if one of the vitreous colonies is planted in broth, a growth results which possesses a great resistance to the lytic agent. Therefore, by merely allowing the normal culture of *Bacillus coli* to age, we have realized an artificial selection of more resistant organisms.

At the same time we have had the good fortune to isolate from a subculture of the original strain a colony of organisms which are, on the other hand, extremely sensitive to the lytic agent. Hence two types of *Bacillus coli* were isolated—one very resistant (Strain R) and the other very sensitive (Strain S)—both artificially selected from the original culture in which they coexisted. The difference in the susceptibility of the two strains is illustrated in the following experiments.

Experiment 2.—1 drop of the lytic agent was spread on the 3 hour slanted agar growth of Type S. With the exception of only two or three colonies, an almost complete clarification occurred (Figs. 2, *A* and 3, *A* and *B*).

With Type R, however, the clarification was transient and soon afterward the path left by the drop was covered with a multitude of minute colonies (Fig. 2, *B*) which, by confluence, overgrew again the surface previously clarified (Fig. 3, *C* and *D*).

Experiment 3.—Broth of varying hydrogen ion concentrations and containing a few drops of lytic agent was seeded respectively with Types S and R. Type R grew luxuriantly, especially in acid medium (pH 6.8), while Type S showed no growth, even in an acid broth.

In addition to the differences in their susceptibility to the lytic agent, both types are distinguished by other characteristics. Type S, multiplying more rapidly than Type R, produces promptly in broth a supernatant film with a whitish band, consisting of micro-organisms, adhering to the wall of the tube at the level of fluid surface; Type R shows the same details but only at a much later period of growth. Both types produce indole and ferment carbohydrates, with the exception of saccharose. The fermentation tests were made by means of stab cultures in semisolid agar and gave us the oppor-

[6] We have made similar observations with Shiga cultures.

tunity to observe another striking distinction. The growth of Type S remains close to the line of puncture (Fig. 4, *B*); on the contrary, Type R diffuses uniformly throughout the whole mass of agar (Fig. 4, *A*). This distinction is due to the difference in the motility of the two types of organisms; Type S is non-motile; Type R possesses, on the other hand, an active motility very much like that of the typhoid bacillus. This affords a very useful means of separating the two types when they are mixed: a stab culture is made in one arm of a U-tube containing semisolid plain agar; only the motile *Bacillus coli* diffuses to the other arm of the tube, from which, after a few hours, we are able to recover it in pure culture.

TABLE I.

Type S.				Type R.			
Guinea pig No.	Dose.	Dilution.	Results.	Guinea pig No.	Dose.	Dilution.	Results.
	cc.				cc.		
1	2	Undiluted.	Died after 26 hrs.	6	2	Undiluted.	Died after 10 hours
2	2	1:2	Survived.	7	2	1:2	" " 15 "
3	2	1:4	"	8	2	1:4	" " 12 "
4	2	1:10	"	9	2	1:10	Survived.
5	2	1:20	"	10	2	1:20	"

In general, *Bacillus coli* is not very virulent, and guinea pigs can withstand intraperitoneal injections of large doses of this microorganism. Hence it is rather difficult to measure variation of virulence between different strains of *Bacillus coli*. However, in the following experiment, in which each type of this bacterium was employed, it will be noted that Type R is more virulent than Type S.

Experiment 4.—We injected into the peritoneal cavity of five guinea pigs respectively 2 cc. of a culture of Type S (grown in plain broth, pH 6.8, for 18 hours), using different dilutions, undiluted, 1:2, 1:4, 1:10, 1:20. Similarly five guinea pigs were injected with a culture of Type R. The results of this experiment are shown in Table I.

Autopsies.—Guinea Pig 1 (injected with *coli* S): The peritoneal cavity contained a purulent exudate with fibrinous clumps. Diffuse fibrinous membrane

covering the viscera, especially the liver. Microscopic examination of the exudate showed large numbers of leucocytes, a few containing phagocytosed bacilli. No free bacilli were visible (Fig. 5). The heart's blood yielded a sparse growth of Type S microorganisms.

Four other guinea pigs which died after injections of large doses of Type S have shown the same conditions, and in three of them cultures of the heart's blood remained sterile.

Guinea Pigs 6, 7, and 8 (injected with *coli* R): Only a faint fibrinous pellicle was noted on the surface of the liver. The peritoneal exudate was serosanguineous and on microscopic examination showed numerous bacteria and an occasional leucocyte (Fig. 6). The heart's blood yielded a profuse growth of Type R bacilli.

It appears from these observations that Type R is more virulent and less phagocytable than Type S. The two types of *Bacillus coli* behave differently, therefore, not only on artificial media but also in the animal body, and, furthermore, retain their individuality even after one passage through a guinea pig.

In spite of the sensitiveness of Type S to the lytic agent, we have never observed a complete dissolution of all these organisms in a culture. Among the vast number of bacteria that are spread over an agar slant there are always a few—a dozen at least—that resist the lytic agent and form colonies. We have found that these resistant organisms were not a few individuals of Type R which had contaminated the culture of Type S; they were not motile. At least it proves that all the individuals which constitute a culture of Type S are not similar in so far as their susceptibility to the lytic agent is concerned. We assume that there are all degrees of resistance against the destructive agent, and if a few individuals can withstand the powerful action of the undiluted lytic agent there must be also a few so sensitive that they can be dissolved even by the lytic agent highly diluted, and that between these two extremes, intermediate degrees exist. This deduction is favored by the following experiment.

Experiment 5.—To a number of agar slants seeded with equal quantities of Type S bacilli and maintained for 3 hours at 37°C. was added 1 drop respectively of increasing dilutions of the lytic agent, as follows: undiluted, 10^{-1}, 10^{-2}, 10^{-3}, etc. As the concentration of the active agent diminished, the number of the resistant colonies increased. At a certain point the clarification of the surface was reduced to small irregular zones and finally to small, perfectly circular areas (Fig. 7).

These small areas of clarification represent the few individuals of *Bacillus coli* which are sensitive enough to be dissolved even in a very dilute solution of the lytic agent. Each of them, in turn, becomes a center of regeneration of the active substance, which, diffusing out at the same distance in all directions, produces a small, circular zone of clarification surrounded by a sort of halo.

Relation to the So Called "Colonies of Bacteriophage."

d'Hérelle, who first observed the localization of the dissolution when the lytic filtrate is very dilute, offers a different explanation for the formation of the small zones of clarification. He considers these areas as colonies of the invisible virus which he supposes to be responsible for the dissolution. When the filtrate is pure the individuals of the bacteriophage are so numerous that their colonies are confluent and produce a general lysis. But when the dilution is sufficiently high, each parasite is isolated and produces localized lysis.

He claims this peculiar aspect to be unquestionable proof of the living nature of the bacteriophage because he questions whether a diffusible substance can localize its action at certain points. As we have seen, we can explain this easily if we do not regard a culture of *Bacillus coli* as a homogeneous whole but as made up of organisms of varying resistance to the lytic agent. While d'Hérelle ascribed the localized dissolution to the dilution of the bacteriophage, we are inclined to look upon the dilution as accessory, and to search for the immediate source of the phenomenon in the relative resistance of the colon bacilli. If this is so, we should expect the similar production of small areas of clarification even with undiluted lytic agent on submitting to its action cultures of greater resistance. As will be shown later, this is precisely what we have often observed.

To sum up, the so called "colonies of bacteriophage" can be explained as well by the hypothesis of a lytic agent as by that of a parasite.

In the preceding experiment decreasing quantities of lytic agent were tested on a constant quantity of *Bacillus coli*. On the other hand, in the following experiment, increasing quantities of *Bacillus*

coli were submitted to a constant quantity of highly diluted lytic agent.

Experiment 6.—200 cc. of a 12 hour broth culture of Type S were centrifuged and to the sediment 2 to 3 cc. of broth were added. The mixture was then filtered through sterile cotton; in this way we obtained a perfectly homogeneous suspension with which the following dilutions were made: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}. In large tubes was mixed respectively 1 cc. of each of these *B. coli* suspensions with 0.5 cc. of very dilute (10^{-5}) lytic agent. Immediately afterward 1 cc. of each of these mixtures with 10 cc. of plain agar was plated in Petri dishes and incubated at 37°C. After 12 hours incubation, the surface of the plates was covered with a uniform pellicle of *B. coli* in which appeared small areas of clarification which were evenly distributed and easy to count.

If the lytic agent is a living organism and these areas are colonies of bacteriophage, the number of areas should be approximately the

TABLE II.

Dilution of lytic principle.	Dilution of B. coli.	Experiment 6. No. of spots.			
		a.	b.	c.	d.
10^{-5}	10^{-4}	69	—	—	25
10^{-5}	10^{-3}	249	56	70	220
10^{-5}	10^{-2}	469	167	187	800
10^{-5}	10^{-1}	328	211	332	508
10^{-5}	Undiluted.	142	95	255	320

same on all plates, since we have added to each the same amount of diluted filtrate. Moreover, since the size of a colony usually varies directly with the abundance of its food, and since *Bacillus coli* comprises the nutrient elements in this case, the size of the areas should increase with the quantity of *Bacillus coli*.

But the results of the preceding experiment show otherwise. The number of areas first increases, then reaches a maximum, and finally decreases, as the quantity of *Bacillus coli* is increased (Table II). The size of the areas instead of increasing, on the contrary, decreases.

These results favor the idea that the lytic agent is a diffusible substance. If the areas represent individuals of *Bacillus coli* so sensitive as to be dissolved even by a very weak lytic agent, the num-

ber of these sensitive individuals should increase with the concentration of the *Bacillus coli* emulsion, and the number of spots should then increase proportionately. This is what is noted at the beginning of the curve. But unfortunately a second phenomenon of opposite tendency interferes with the preceding one: when the lytic agent is added to the *Bacillus coli* suspension, it combines partially with proteins which have nothing to do with the dissolution and so decreases the dissolving action in direct proportion to the increase in quantity of *Bacillus coli*.[7] If, on one hand, the clarified areas increase with the increase of the *Bacillus coli* emulsion because the number of sensitive organisms is increasing, on the other hand, these areas tend to decrease because the activity of the lytic agent is decreasing. The combination of these two phenomena of opposite action should give us the curve that we have observed. The evidence at hand leads us to the hypothesis that bacteriophage is a diffusible, rather than a living substance.

Non-Specificity of the Lysis.

The lytic filtrate used in the preceding experiments was specific. While it is active on the *Bacillus coli* used in injecting the guinea pigs, it is without any action not only on other closely related species but also on other strains of *Bacillus coli*. But in the following experiments we have been able to extend the lytic action to other species.

Experiment 7.—With the aim of increasing our stock of lytic agent we added 1 cc. of the original filtrate to each of two flasks, one containing 25 cc. of a young broth culture (pH 8) of Type S and the other of Type R. Dissolution of the growth occurred. After 48 hours incubation, we filtered both cultures and thus obtained besides the original filtrate (Filtrate O), two new filtrates, Filtrate 1 (Type S) and Filtrate 2 (Type R).

With a sample of Filtrate 2 a very marked dissolution was observed[8] of Shiga bacilli, Flexner bacilli, Hiss Y type of Flexner bacilli, and also a strain of *B. coli*

---

[7] This phenomenon is similar to the one observed by Loeb (Loeb, J., Artificial parthenogenesis and fertilization, Chicago, 1913, 145) on the parthenogenetic fertilization of eggs with small quantities of acids. Very dilute solutions of acid are able to fertilize a small number of eggs, but not a larger number. The acid in combining with the jelly of the eggs loses a certain part of its activity; it still retains enough efficiency when the eggs are not numerous, because the total quantity of jelly is small, but loses it completely, on account of the abundance of jelly, when the number of eggs is great.

[8] These observations were made by Dr. Martha Wollstein.

communis which was unattacked by the original filtrate. This observation led us to control the action of all three filtrates on agar slants, seeded with the following bacteria: Types S and R, both isolated from the original culture of Bordet and Ciuca, *B. coli communis*, *B. coli communior*, *B. dysenteriæ* Shiga, *B. dysenteriæ* Flexner, Hiss Y type of Flexner bacilli, *B. typhosus*, and *B. paratyphosus* A and B.

Filtrate O, the original lytic agent, had only a weak lytic action, limited to Types S and R; Filtrate 1 was similarly weak, but produced a few small areas of clarification on the three dysenteric bacilli; Filtrate 2, on the other hand, was extremely active and produced a complete dissolution of Type S, an almost complete dissolution of Type R and of the three dysenteric bacilli, and a complete clarification of the strain of *B. coli communis*. Only the *B. coli communior*, the typhoid, and both paratyphoid strains were unaffected.

Our supposition was to attribute this increase of the power of the lytic agent to the fact that Filtrate 2 was prepared with a more re-

TABLE III.

Fil-trate.	B. coli.				Dysenteric bacilli.			Ty-phoid.	Paratyphoid bacilli.	
	Type S.	Type R.	Com-munis.	Com-munior.	Shiga.	Flexner.	Hiss Y.		A.	B.
O	+++	++	—	—	. —	—	—	—	—	—
1	+++	++	—	—	+	+	+	—	—	—
2	++++	+++	+++	—	+++	+++	+++	—	—	—
3	++++	+++	+++	—	++++	++++	++++	+	—	—
4	+++	++	+	—	++	+++	+++	+++	—	++

++++ indicates complete clarification, no resistant colonies; +++, almost complete clarification, less than twelve resistant colonies; ++, moderate degree of clarification, many resistant colonies; +, only a few small areas of clarification;—, negative result.

sistant strain, Type R. The following experiment demonstrates that this is the actual condition and the use of a resistant strain gives a method of increasing the efficacy of the lytic agent and extending its action to the other species as yet refractory.

Experiment 8.—By allowing 1 cc. of Filtrate 2 to act for 48 hours on 25 cc. of a young culture of Type R bacilli, we have obtained a still stronger filtrate (Filtrate 3) which, fortunately, produced four or five minute areas on a slant of typhoid bacillus. Following this last indication, we mixed 25 cc. of a young culture of typhoid bacillus with 3 cc. of Filtrate 3, and after 48 hours incubation, obtained a filtrate (Filtrate 4) extremely lytic for typhoid, as well as for para-

typhoid B bacilli. At the same time, the action of Filtrate 4 on *B. coli* was diminished to a certain extent. Table III shows the activity of the different filtrates.

It is obvious that we can now multiply similar combinations by allowing one or another of our filtrates to act on a well selected strain as intermediary. It will be of interest to study the relation between different microbic species in this respect, and to note to what extent the lytic activity can be transmitted from one species to another.

CONCLUSIONS.

The inhibition produced by the lytic agent on the growth of *Bacillus coli* is greatly influenced by the reaction of the medium; it is faint in a slightly acid (pH 6.8) or neutral (pH 7) or even slightly alkaline broth (pH 7.4), but is much stronger in a more alkaline medium (pH 8 or 8.5).

We have isolated from the original strain of *Bacillus coli* two types of organisms; one (Type S) is sensitive to the lytic agent, the other (Type R) is much more resistant. These types are distinguished also by other characteristics: Type S grows quickly in artificial medium and is non-motile; Type R grows more slowly, is extremely motile, much less phagocytable, and more virulent. Both types produce indole and ferment carbohydrates, with the exception of saccharose. Both types keep their individuality even after passage through a guinea pig.

We have also demonstrated that even a culture of a single type, Type S for instance, is not a homogeneous whole but is made up of organisms of varying resistance to the lytic agent; only a few are resistant enough to overcome the strong action of the undiluted lytic agent. On the other hand, only a few as well are sufficiently sensitive to be dissolved even by very dilute lytic agent.

This explains why dilute lytic agent spread on an agar plate seeded with *Bacillus coli* confines its action only to certain places and produces the small round areas of dissolution that d'Hérelle considered as "colonies of bacteriophage." Moreover, we have observed the same localized action even with non-dilute lytic agent when submitting to its action cultures of greater resistance.

Our original lytic agent was found to be specific; it acted exclusively on the *coli* with which the guinea pigs were injected. By allowing this original lytic principle to act on broth cultures of our two types of *Bacillus coli*, we have obtained two new filtrates. The first, resulting from dissolution of the sensitive Strain S, is specific as is the original filtrate. But with the second, obtained from the resistant Strain R, Dr. Wollstein has found a marked action on Shiga, on Flexner, and on Hiss dysentery bacilli. In consequence of this observation, we have been able, by a method of successive passages through appropriate strains, to extend the lytic power to other species, as typhoid and paratyphoid bacilli, and have obtained by this somewhat different technique results similar to those recently published by Bordet and Ciuca.

EXPLANATION OF PLATES.

PLATE 5.

FIG. 1. Tube A: Experiment of Bordet and Ciuca. A young culture of *B. coli* on slanted agar covered with lytic agent. Only a few organisms resist dissolution and produce irregular colonies. Tube B: Agar slant culture of *B. coli*, 6 weeks old. Note on the uniformly dull film of the desiccated culture a few hyaline colonies which have resisted desiccation (*coli* R).

FIG. 2. Experiment 2. 1 drop of lytic agent placed on 3 hour cultures of *B. coli* (Types S and R). Results after 6 hours incubation at 37°C. Tube A: Type S (sensitive). The path of the drop is free from growth. Tube B: Type R (resistant). The path of the drop is already covered with a great number of minute resistant colonies.

FIG. 3. The same experiment. Results after 24 hours incubation. Tubes A and B: *Coli* S. Only a few resistant colonies may be seen. Tubes C and D: *Coli* R. Numerous resistant colonies have overgrown the area previously clarified.

PLATE 6.

FIG. 4. Stab cultures of Types S and R in semisolid agar. Tube A: Type R (motile). The growth diffuses evenly throughout the whole mass of agar. Tube B: Type S (non-motile). The growth remains close to the line of puncture.

FIG. 5. Peritoneal exudate of a guinea pig dead after injection of *coli* S. Numerous leucocytes. No free bacteria.

FIG. 6. Peritoneal exudate of a guinea pig dead after injection of *coli* R. No leucocytes. Numerous free bacteria.

FIG. 7. Action of increasing dilutions of lytic agent on *B. coli* S.

(Gratia d Hérelle phenomenon)
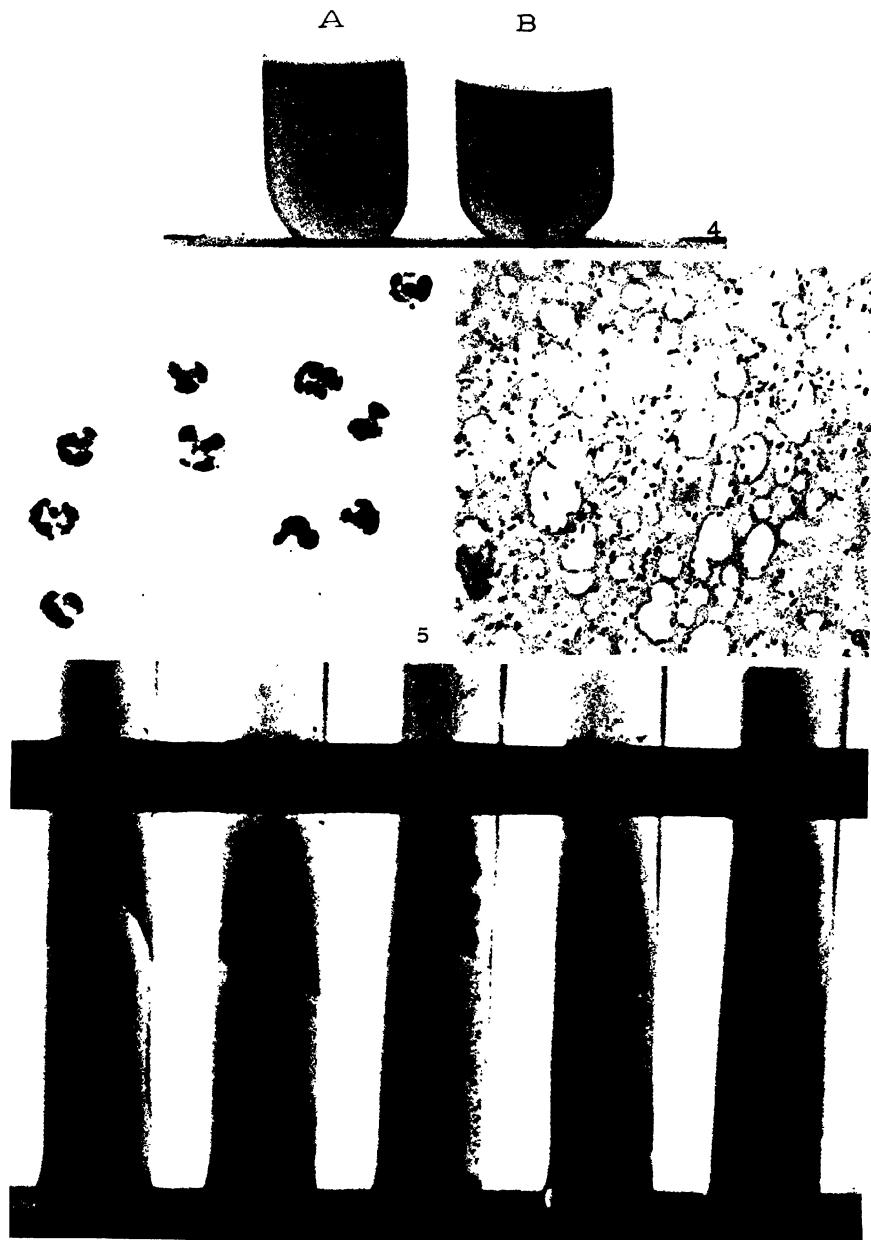
(Gratia: d'Hérelle phenomenon.)

PROPHYLAXIS AND SERUM THERAPY OF YELLOW FEVER.

By HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

Experimental transmission of yellow fever to animals and the isolation of *Leptospira icteroides* was accomplished in Guayaquil in 1918,[1] and repeated confirmations have since been made. During December, 1919, and January, 1920, Noguchi and Kligler, working in Mérida, Yucatan, succeeded in transmitting yellow fever to guinea-pigs and isolating *Leptospira icteroides*,[2] and again in northern Peru later in 1920.[3] In the summer of the same year, in Vera Cruz, Perez-Grovas[4] and his coworkers were able to produce experimental yellow fever and obtain cultures of *Leptospira icteroides*.

The latest confirmation (January, 1921) comes from Le Blanc of The Rockefeller Institute, who has also successfully transmitted the disease from a patient in Vera Cruz to guinea pigs and has obtained a culture of *Leptospira icteroides*. The cultures obtained by Perez-Grovas and by Le Blanc have been sent to me, and I have found them to be identical with other strains of *Leptospira icteroides*. Gastia-

---

1. Noguchi, Hideyo: Etiology of Yellow Fever, Papers I–XII, J. Exper. Med. **29**: 547–596 (June) 1919; **30**: 1–29 (July) 1919; **30**: 87–93 (Aug.) 1919; **30**: 401–410 (Oct.) 1919; **31**: 135–168 (Feb.) 1920; **32**: 381–400 (Oct.) 1920.

2. Noguchi, Hideyo, and Kligler, I. J.: Experimental Studies on Yellow Fever Occurring in Mérida, Yucatan, J. Exper. Med. **32**: 601 (Nov.) 1920; Immunological Studies with a Strain of Leptospira Isolated from a case of Yellow Fever in Mérida, Yucatan, ibid. **32**: 627 (Nov.) 1920.

3. Noguchi, Hideyo, and Kligler, I. J.: Experimental Studies on Yellow Fever in Northern Peru, J. Exper. Med. **33**: 239 (Feb.) 1921; Immunology of the Peruvian Strains of *Leptospira icteroides*, ibid. **33**: 253 (Feb.) 1921.

4. Perez-Grovas, P.: Experimental Transmission of Yellow Fever, J. A. M. A. **76**: 362 (Feb. 5) 1921.

buru,[5] of the Instituto de Higiene of Lima, transmitted yellow fever to guinea-pigs from cases occurring during an epidemic in Piura, Peru, in 1919. Stimson,[6] of the U. S. Public Health Service, had demonstrated in 1907 the presence of a spiral organism, now identified as *Leptospira icteroides*, in a preparation, stained by the Levaditi method, of the kidney from a yellow fever patient dying during the epidemic in New Orleans in 1905.

The symptoms and lesions produced in animals by the inoculation of *Leptospira icteroides*[7] closely parallel those of human yellow fever, and comprise mainly jaundice, hemorrhages and intense acute nephritis. In the guinea-pig the degree of hemorrhage is very marked in the lungs and on the serous surfaces of the stomach and intestines, while minute hemorrhages of various dimensions occur almost constantly from the mucosa into the gastric cavity or intestinal canal. In young dogs the hemorrhages of the lungs are generally slight, but the hemorrhages from the mucosa of the gastro-intestinal tract, producing so called "black vomit" and melena, make the picture almost identical with that of yellow fever in human beings. The lesions produced in the marmoset, the only species of monkey which has so far been found to be susceptible to experimental infection with *Leptospira icteroides*, are intermediary between those of the guinea-pig and those of the dog. While the distribution and degree of hemorrhage in the different species of animals varies, the more or less pronounced fatty degeneration of the parenchymatous cells of liver and kidney is always present.

The specific immunity reaction for *Leptospira icteroides* demonstrated with the serums of yellow fever convalescents in Guayaquil,[8] Mérida,[2] and Peru[3] constitutes further proof of the etiologic relation of this organism to yellow fever. The existence of this property in convalescent serum led to a study of the possibility of preparing a

5. Gastiaburu: Personal communication to the author.

6. Stimson, A. M.: Note on an Organism Found in Yellow Fever Tissue, Reports, U. S. P. H. S. 22, Part 1, 541, 1907.

7. Noguchi, Hideyo: Symptomatology and Pathological Findings in Animals Experimentally Infected, J. Exper. Med. 29: 547 (June) 1919.

8. Noguchi, Hideyo: Properties of Blood Serum of Yellow Fever Patients in Relation to *Leptospira icteroides*, J. Exper. Med. 30: 9 (July) 1919.

specific immune serum for the treatment of yellow fever and to a consideration of the effect of the inoculation of killed cultures of *Leptospira icteroides* as a means of protection against the disease.

Serum Treatment of Yellow Fever.

The Serum Treatment of Animals Experimentally Infected.—Preliminary therapeutic experiments with an immune serum,[9] prepared in the horse, in experimental yellow fever in guinea-pigs showed that the serum had definite value in checking the progress of the infection and that it was capable, when administered during the period of incubation, of completely preventing the development of the disease. Moreover, when used in the early stages of infection it modified the course of the disease and prevented a fatal outcome. At a later stage, however, when jaundice and nephritis had been present from two to three days and the animal was near collapse, the serum had no perceptible beneficial effect.

The strength of the serum was such that, when injected simultaneously in a quantity as small as 0.001 cc., it had the power to neutralize 5,000 minimum lethal doses of *Leptospira icteroides*, that is, 1 cc. of the serum was capable of neutralizing 5,000,000 minimum lethal doses. It was found that infection could be prevented when 0.1 cc. of the serum was injected at any time during the period of incubation (i.e., within seventy-two hours after inoculation). After onset of fever, that is, ninety-six hours after inoculation, the same amount of serum either arrested the progress of the infection or modified its course to that of a nonfatal infection. On the other hand, as much as 1 or 2 cc. of the serum failed to prevent fatal termination of the disease when administered after the animals had reached the stage of intense jaundice, descending temperature, hemorrhages and nephritis, and were nearing collapse.

Serum Treatment of Yellow Fever in Man.—The serum was first tried in the treatment of human yellow fever in September, 1919, when an American marine on the U. S. S. *Chicago*, off Honduras, was treated by Gen. T. C. Lyster and Dr. W. Pareja. The next patient

9. Noguchi, Hideyo: Serum Treatment of Animals Infected with *Leptospira icteroides*, J. Exper. Med. 31: 159 (Feb.) 1920.

treated was the Mexican minister to Nicaragua, also by Drs. Lyster and Pareja. The results in these two cases were regarded by Lyster and Pareja, as well as by General Gorgas, who was also present, as definitely indicative of the value of the serum, and led to the treatment of other cases. The total number of treated cases up to Dec. 31, 1920, is 170 (Table I).

TABLE I.

Analysis of One Hundred and Seventy Cases of Yellow Fever Treated with Anti-Icteroides Serum.

	Treated on or before 3d Day			Treated after 4th Day		
	Total	Recovered	Died	Total	Recovered	Died
Group 1:						
Salvador, 1920...............	14	11	3*	28	15	13
Guatemala, 1920..............	3	3	0	1	0	1
Honduras, 1919..............	1	1	0	..	..	..
Mexico, 1920						
Mérida....................	4	4	0	4	0	4
Vera Cruz.................	16	16	0	3	0	3
Gutierrez Zamora..........	17	17	0	1	0	1
Peru, 1920.................	4	3	1†	..	..	..
Total, Group 1...........	59	55	4 (6.7%)	37	15	22 (59%)
Group 2:						
Tuxpan, Mexico, 1920........	36	27	9 (25%)	38	21	17 (45%)
Total, both groups...........	95	82	13 (13.6%)	75	36	39 (52%)

* Total of 20 cc. in two days, begun on the third day, undoubtedly too small to have any material effect (Dr. Bailey).

† Patient subjected to forced journey during first two days of illness; disease extremely severe; patient exhausted, with severe nephritis, when admitted to the hospital.

During a small epidemic occurring near Los Amates, Guatemala, in July, 1920, the serum was employed by Dr. Vaughn in four cases, in three of them on or before the third day of illness, in the other later. The three patients treated early all recovered, the other died.

In Salvador, forty-two patients were treated with serum by Dr. Bailey, fourteen on or before the third day of illness. Three of the fourteen, however, received a quantity of serum which, Dr. Bailey states, was "too small to have any material effect" (20 cc. in the course of the third and fourth days). These three patients died; the other eleven all recovered.[10] Twenty-eight patients were treated later than the fourth day of illness, and in these cases the usual high
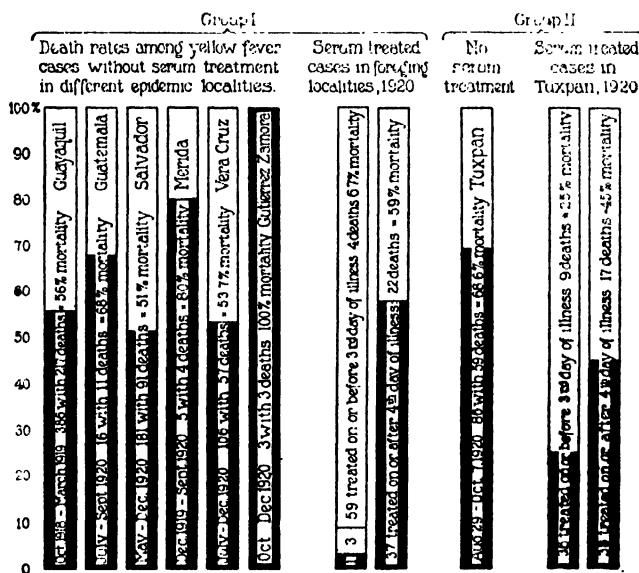
Chart 1. Death rates among yellow fever patients with and without serum treatment.

mortality rate of yellow fever (46 per cent in this instance) was apparently unaffected by the use of the serum. In Mérida, eight cases have been treated by Drs. Hernandez, Lara and Villamil, who report four recoveries among four cases treated with serum before the third day and four deaths among those treated later. In Vera Cruz, in 1920, sixteen cases were treated with serum on or before the third day without any mortality, while three patients treated after

10. It has been recommended that when there is no danger of anaphylaxis, 60 cc. of serum be given intravenously at the earliest opportunity.

the third day all died. In Gutierrez Zamora, Mexico, eighteen patients were treated with the serum. All recovered except one patient who did not receive serum until the end of the fourth day of illness. In the same town there were three cases seen before the third day of illness but not treated with serum; these three patients died. In Peru, four cases were treated early in the disease, with one death. This last case was exceptional, however, in that the boy had been brought through a tropical desert after onset (a journey of thirty-six hours) and had severe nephritis when admitted to the hospital. In Tuxpan, Mexico (Table I, Group 2) seventy-four cases were treated with serum, thirty-six on or before the third day. The results reported from Tuxpan are the least favorable of all those reported—nine deaths and twenty-seven recoveries (25 per cent mortality). The remaining thirty-eight cases were treated later than the third day, with twenty-one recoveries and seventeen deaths (45 per cent. mortality).

An examination of the results of serum treatment in various localities shows a general agreement among them: the earlier serum is given, the lower the mortality. Exclusive of the Tuxpan series, fifty-nine patients were treated on or before the third day; of these, fifty-five recovered and four died. It has already been explained that in three of the four cases in which death occurred, the total amount of serum administered during a period of two days was 20 cc.; the fourth case is that of the Peruvian boy who was subjected to a long journey after the onset of fever. The total mortality in this series was, therefore, 6.7 per cent. In Tuxpan the mortality among cases treated early was 25 per cent. (seventeen were treated on the third day alone, with 19 per cent. mortality), while the death rate of later treated cases was 45 per cent. The reduction in mortality among cases treated on or before the third day is undeniable. On the other hand, the serum does not seem to exhibit any beneficial effect on the course of the disease when given after the fourth day of illness.

For comparison there are given in Table II the death rates of cases not treated with serum during the various epidemics. They vary according to the number of cases included in the statistics— the fewer the cases, the less reliable the percentages. The lowest rate was in Salvador (51 per cent.), the highest in Mérida (80 per

cent.) and Gutierrez Zamora (100 per cent.). Comparison of the mortality rates among these untreated cases with those of cases treated with serum on or before the third day brings out a great difference in favor of the treated cases which is difficult to explain on the ground of accidental coincidence. Apparently the serum actually helped to cut the infection short before it had caused irreparable injury to the organs, particularly the kidneys. Once such injuries have been done, however, as is usually the case in severe yellow fever by the fourth day of illness, an antimicrobial serum cannot be expected to effect any restoration. In certain of these cases, however, a marked improvement has been reported to have occurred after the

TABLE II.

Death Rates in Various Localities among Yellow Fever Cases Not Treated with Serum.

	Cases	Deaths	Mortality Percentage
Guayaquil, Oct., 1918–Mar., 1919	386	217	56.0
Guatemala, July–Sept., 1920	16	11	68.0
Salvador, May–Dec., 1920	181	91	51.0
Mérida, Dec., 1919–Sept., 1920	5	4	80.0
Vera Cruz, July–Dec., 1920	106	57	53.7
Gutierrez Zamora, 1920	3	3	100.0
Tuxpan, Aug. 29–Oct. 7, 1920	86	59	68.8
Total	783	442	56.4

administration of serum, owing probably to the fact that parenchymatous disorganization was proceeding slowly.

Vaccination against Yellow Fever.

Vaccination of Experimentally Infected Animals.—Early experiments showed that the injection of killed cultures of *Leptospira icteroides* into susceptible animals conferred on them a state of immunity which rendered them resistant to subsequent inoculation with virulent cultures.[11] The duration of this immunity has been found

11. Noguchi, Hideyo, and Pareja, Wenceslao: Prophylactic Inoculation Against Yellow Fever, J. A. M. A. 76: 96 (Jan. 8) 1921.

to be at least five to six months; the maximum duration is yet to be determined.

Vaccination of Human Beings.—The absence of any ill effects following the injection of killed cultures into animals led to the application of a similar procedure to human beings. The results of the early experiments in Guayaquil have already been reported.[11] The potency of the vaccine available at that time was rather low (2,000,000 leptospiras per cubic centimeter, as compared with 2,000,000,000, which represents the concentration of the preparation
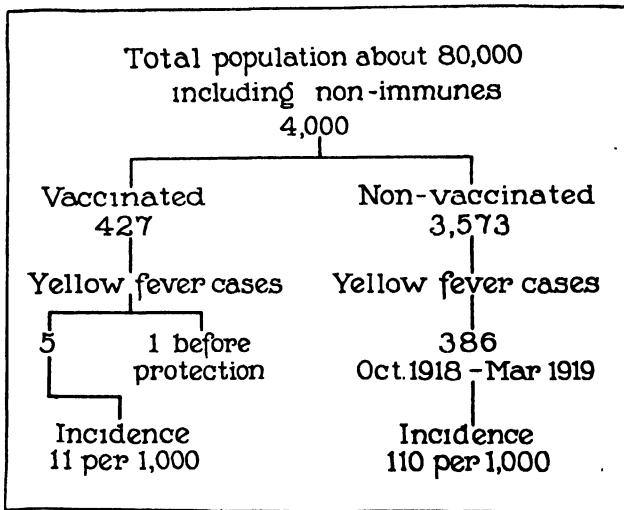
Chart 2. Yellow fever vaccination, Guayaquil, Ecuador, 1918–1919.

now used), yet its protective effect was undeniable. No cases of yellow fever developed among those persons who received 2 cc. of vaccine. The number of nonimmunes in Guayaquil was not definitely known, but a liberal estimate would place it at about 4,000. Of these, 427 were vaccinated, of whom five later contracted yellow fever—a morbidity rate of 11 per thousand. During the same period (October, 1918, to March, 1919) there were 386 cases of yellow fever among the unvaccinated, a rate of incidence of 110 per thousand (386:3,577) (Chart 2).

The strength of the vaccine, as already noted, has since been greatly increased, and preparations of many times the concentration of that used in Guayaquil have been employed by Lyster, Bailey and Vaughn in Guatemala and Salvador, Vasconcelos, Casasús, Lynn and others, in Mexico, and by the department of health of Peru. The additional number of persons vaccinated is about 7,500. The vaccine has been given in two subcutaneous injections of 2 cc. each, when possible, although, as the table shows, circumstances often prevented the administration of the second injection. The results in all vaccinations to Dec. 31, 1920, are summarized in Table III.

The incidence of yellow fever among the vaccinated may be con-

TABLE III.

Vaccination against Yellow Fever in Man.

	Number Vaccinated	Number Receiving Two Injections
Guayaquil, 1918–1919................................	427	.....
U. S. S. Chicago, 1919...............................	75	75
Marines and others, 1919............................	250	250
Amapala, 1919.......................................	175	175
Salvador, 1920......................................	3,607	138
Guatemala, 1920.....................................	1,383	592
Tuxpan, Mexico, 1920................................	2,000	2,000
Tambogrande, Peru, 1920............................	47	.....
Total...	7,964	3,230

sidered in two groups: (*a*) those in whom the disease developed within a comparatively short time—from one to ten days after the time of the last inoculation, and (*b*) those in whom infection took place one month or longer after vaccination. Since the longest incubation period in yellow fever in man, as experimentally determined by the French Yellow Fever Commission in Brazil, is twelve days, and the average from three to six days, the group of vaccinated individuals under Group *a*, that is, those who contracted yellow fever within from one to ten days after vaccination, must be excluded from a consideration of the protective effect of the vaccination. On the other hand, the occurrence of yellow fever among vaccinated persons

after the lapse of one month or longer would indicate inefficacy of vaccination as a prophylactic measure. As the statistics given in Table III show, 3,230 persons received two inoculations of the vaccine; among these all the cases which occurred fall in the *a* group. There were eighteen such cases, all of which showed the symptoms of yellow fever within from one to ten days after the last inoculation. The remaining 4,734 persons received only one injection of vaccine; among the number, five typical cases of yellow fever (Guayaquil, 1918–1919[11]) and five in which the symptoms were suspicious (Dr. Bailey's report from Salvador, 1920) arose in from one to three months. It has already been noted that the vaccine employed in Guayaquil was several hundred fold less strong than that used later in Central America and Mexico. In addition to these ten cases there were also, in the single vaccination group, eight persons in whom yellow fever developed within from two to ten days after vaccination (one in Guayaquil, five in Salvador, one in Guatemala, and one in Tambogrande, Peru).

The epidemic of yellow fever in Salvador broke out in May, 1920. Vaccination was begun in August, and up to Dec. 31, 1920, 3,607 persons in the infected areas had been vaccinated, 138 of whom had received two injections of vaccine. The total number of nonimmunes in the ten infected districts was, according to Dr. Bailey's estimate, 113,000. Among those who received two injections of the vaccine, no case of yellow fever occurred. In the group receiving a single injection, however, there occurred, within ten days after vaccination, as already mentioned, five cases which Dr. Bailey regarded as suspicious of yellow fever, and, still later, five more suspicious cases. Of the latter patients, three died and came to necropsy, but the diagnosis of yellow fever was not definitely confirmed. During the same period 181 cases of yellow fever occurred among the nonvaccinated population (Chart 3).

In Guatemala, in the small town of Los Amates, with about 660 inhabitants, there were twelve cases of yellow fever from June to August, 1920. Vaccination was begun in August, and 617 vaccinations were carried out between August 20 and August 30. No case of yellow fever developed among the vaccinated, and only one among the few left unvaccinated at Los Amates. The remainder of the

nonimmune population was vaccinated, September 18, after which date no further case occurred. At Virginia one case of yellow fever was discovered, September 7, and the entire population (350) was vaccinated, September 26–27. No further case occurred. In Quirigua and Puerto Barrios, although no cases of yellow fever had occurred, 105 persons were vaccinated. No case of yellow fever developed. The total number of vaccinations in Guatemala was 1,383, and 138 persons received two injections (Chart 4).

In Tuxpan, Mexico, with about 6,000 inhabitants, eighty-six cases of yellow fever occurred during a period of thirty-nine days from Aug. 20 to Oct. 7, 1920, a morbidity rate of 14 per thousand. Vaccination was begun, October 7, and during the subsequent twenty-

Yellow Fever Vaccination
Salvador, 1920

Approximate population of ten infected localities
about 113,000

3,607	109,000
(Vaccinated-immediate danger zone)	Non-vaccinated
(Aug. 13 - Dec. 31, 1920)	Y. F. cases
5 suspicious cases (Y. F. doubtful) 5 suspicious, but unconfirmed cases. developed before protection.	181 (May-December, 1920)
Incidence 0	Incidence 1.6 per 1000
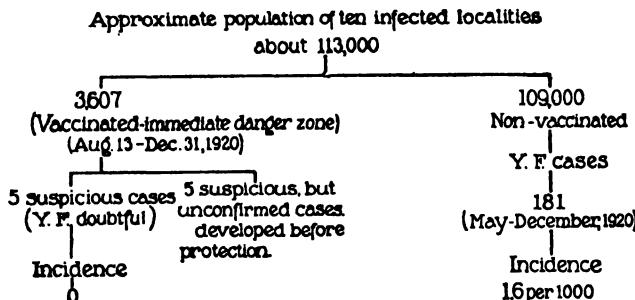
Chart 3. Yellow fever vaccination, Salvador, 1920.

nine days (October 7 to November 5) 2,000 nonimmune persons were vaccinated, among whom seventeen contracted yellow fever within from one to six days after vaccination, that is, there was a high rate of morbidity among vaccinated individuals during the period prior to the establishment of the immunity. Among the vaccinated who escaped infection within the week following the last inoculation, no cases developed during the subsequent period of the epidemic, which ceased in December, 1920. On the other hand, during a period of eighty-five days (October 7 to December 31) eighty-five cases occurred among the unvaccinated group, fifty-seven of them between October 7 and November 21, and twenty-eight

between November 22 and December 31.   Hence the morbidity rate
among the 4,000 unvaccinated during the period of eighty-five days
was 21.3 per thousand. The fact that no cases of yellow fever
occurred among vaccinated persons after the lapse of one week
following vaccination, while the epidemic had remained unabated in
intensity, as shown by the incidence among the unvaccinated during
the same period, seems to indicate a definite protective effect of
vaccination (Chart 5).

In the small town of Tambogrande, in northern Peru, with 500
inhabitants, two isolated cases of yellow fever occurred. Forty-
seven persons in the neighborhood of the primary focus were subse-
quently vaccinated (Dr. Valcárcel). Within two weeks after vacci-
nation, eighteen new cases arose among the villagers; one of these
persons had been vaccinated, but only ten days previous to the onset
of disease.

Comment.

It is understood that vaccination constituted only a part of the
campaign against yellow fever, and in all these localities it was car-
ried out simultaneously with the antistegomyia campaign. Just
what part the vaccination played in checking the further spread
of the disease is therefore not easily estimated; but one fact stands
out, namely, that practically all persons vaccinated escaped yellow
fever, notwithstanding the opportunities for infection to which both
vaccinated and unvaccinated were alike exposed under otherwise
identical conditions, as shown by the fact that during the period of
ten days following vaccination, before the protective effect of the
vaccine could have developed, the number of victims of yellow fever
among vaccinated and unvaccinated was equally great. Vaccina-
tion, which protects the nonimmune person from infection, is a
valuable weapon in itself, although it does not supplant the method
of elimination of yellow fever by the antistegomyia campaign.

SUMMARY.

The transmission of yellow·fever from man to guinea-pigs and the
isolation of *Leptospira icteroides* have been repeatedly accomplished

by various workers in the course of different epidemics; in Guayaquil by Noguchi (1918); in Mérida and Peru by Noguchi and Kligler (1919–1920); in Piura by Gastiaburu (1919); in Vera Cruz by Perez-Grovas (1920) and by Le Blanc (1921).

Anti-icteroides serum reduces the mortality in yellow fever when used on or before the third day of the disease. Of 170 cases, ninety-five have been treated on or before the third day, with thirteen deaths (13.6 per cent. mortality), while the average death rate of untreated patients during these epidemics has been 56.4 per cent. (442 deaths among 783 cases not treated with serum). On the other hand, treatment with serum after the fourth day has no appreciable
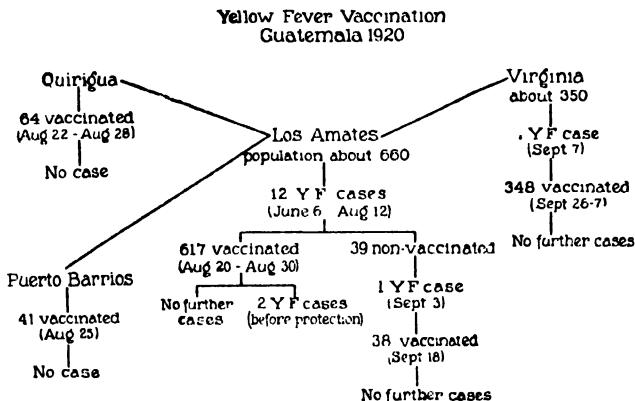
Chart 4. Yellow fever vaccination, Guatemala, 1920.

effect, since there were thirty-nine deaths among seventy-five cases (52 per cent. mortality).

Prophylactic inoculation by means of the injection of 2 cc. of the killed culture of *Leptospira icteroides* (containing at least 2,000,000,000 organisms per cc. is of definite protective value. Among 3,230 persons vaccinated twice, no case of yellow fever developed, while 267 cases occurred among the nonvaccinated (Guatemala, Salvador, Tuxpan) notwithstanding the fact that both groups of individuals were equally exposed to infection. Among 4,307 persons receiving only a single inoculation of the vaccine, only five suspicious cases (Salvador) developed.

The protection resulting from vaccination does not become effective until about ten days after the last injection, as shown by the frequent case incidence occurring among the vaccinated population within from one to ten days after they were vaccinated. There were twenty-three such cases among 7,537 persons who were vaccinated with the standard vaccine.

Tuxpan, Mexico, 1920

Total population about 6,000

86 Yellow fever cases
(Aug. 29 - Oct. 7, 1920)

Incidence
14 per 1,000

2,000
vaccination
(Oct. 7, - Nov. 5, 1920)

Yellow fever cases

0   17
(before protection)

Incidence
0

4,000
non-vaccinated

Yellow fever cases

85
(Oct. 7, - Dec. 31, 1920)

Incidence
21.3 per 1,000
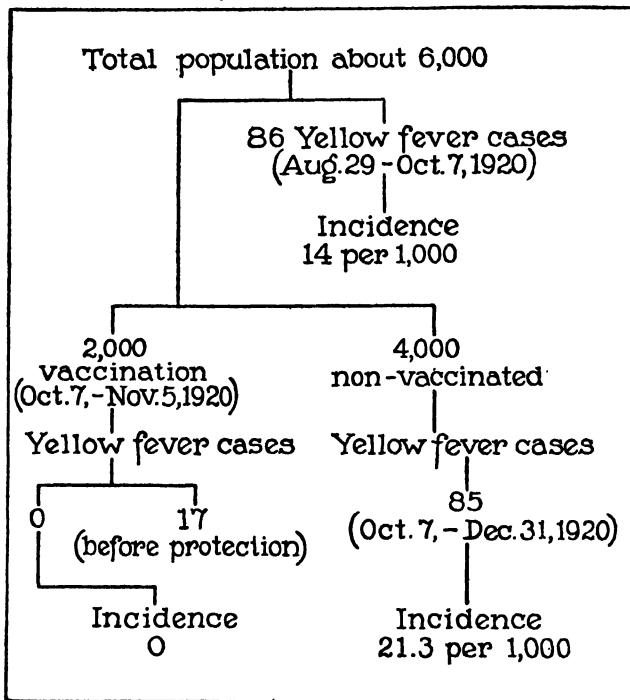
Chart 5. Yellow fever vaccination, Tuxpan, Mexico, 1920.

NOTE.—The records of vaccination and serum treatment presented here comprise the work of a number of observers. For statistics from Central America I am indebted first to Dr. Theodore C. Lyster and Dr. Wenceslao Pareja, and then to Dr. Charles A. Bailey (Salvador) and Dr. Emmett I. Vaughn[12] (Guatemala); for the records of

12. Dr. Lyster and his associates are intending to present their own experiences in a paper to be prepared by them.

Mexican cases to Dr. A. B. Vasconcelos[13] and Dr. Graham Casasús of the Consejo Superior de Salubridad, and to Dr. Diego Hernandez of the Junta de la Sanidad de Yucatan; also to Dr. T. J. Le Blanc of the staff of the Rockefeller Institute, who has been working in Vera Cruz; the Tuxpan statistics were furnished by Dr. W. J. Lynn and Dr. Guadarrama, the work in Ecuador and Peru was done with the cooperation and assistance of the Ecuadorean and Peruvian health authorities.

13. Dr. Vasconcelos's details regarding results in Mexico are soon to appear in print.

NOTE ON THE PRESERVATION OF STOCK STRAINS OF TREPONEMA PALLIDUM AND ON THE DEMONSTRATION OF INFECTION IN RABBITS.

BY WADE H. BROWN, M.D., AND LOUISE PEARCE, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

The maintenance of stock strains of *Treponema pallidum* for teaching or experimental purposes has been both time-consuming and expensive, due to the necessity for constant watchfulness and frequent transfer from animal to animal in order to guard against loss of strains, but no way has been found to overcome these difficulties, and in spite of the greatest care, valuable strains are frequently reported as lost.

In like manner, the usefulness of *pallidum* infections in rabbits for experimental purposes has been subject to serious limitations on account of the lack of reliable means for determining end-results as regards infection when no demonstrable lesions were present. This has been especially true in chemotherapeutic investigations in which the method for determining a cure, and the only one available, was more or less prolonged clinical observation. It has been assumed that healing of existing lesions without recurrence within a short period of time (1 to 3 months) constituted evidence of cure and the results of all experiments thus far reported have been based upon the use of criteria of this kind.

The supposed necessity for frequent transfers, the presumed loss of stock strains of *Treponema pallidum*, and the supposition that freedom from lesions constituted evidence of cure were all based upon the belief that *pallidum* infections in the rabbit are self-limiting— that with the healing of the lesions the infection also became extinct. Numerous isolated observations by ourselves and others have cast considerable doubt upon the validity of these earlier views, but until quite recently, no systematic experiments were carried out for the

259

purpose of determining the exact nature of the infection in the rabbit as regards dissemination of organisms, the duration of the infection, and the possibilities of recovering the virus from animals after all manifestations of disease had disappeared.

During the past 2 years, a large series of experiments has been carried out in an attempt to obtain definite information upon these points. One of the methods employed was that of test inoculations of normal animals with material from superficial lymph nodes of infected animals. Parts of this work have been reported in connection with studies on the dissemination of spirochetes (1, 2) and the demonstration of spirochetes in the lymph nodes during latent periods of infection (3).

The details of these experiments need not be repeated; it will suffice to state that test inoculations have been made from inguinal or popliteal nodes of 51 rabbits with positive results in all instances. The material studied included four classes of anmals: (1) animals with developing or active infections of from 48 hours to $2\frac{1}{2}$ years duration; (2) animals with latent infection in which no lesions had been present for from 3 months to 2 years with a period of infection ranging from 7 months to 4 years and 3 months; (3) drug-treated animals in which no lesions had recurred during a period of 3 months observation; (4) animals used for serial passage of *Treponema pallidum* from lymph node to testicle over a period of about 14 months. The majority of the tests were carried out during the first 3 months of the infection.

Before it could be concluded that the organisms recovered were localized in the tissues, it was necessary to exclude the blood as a possible source. During early stages of the infection, this could be done only upon the basis of relative infectivity. Later, however, blood inoculations were uniformly negative while the lymph nodes gave positive results.

The experiments cited showed that with old strains of *Treponema pallidum*, generalization and localization of the organisms in lymphoid tissues are a constant phenomenon of the infection; they also indicated that the infection is permanent and that the treponema can be recovered at any time by inoculation of material from superficial lymph nodes of infected animals. It thus appears that while rabbits acquire a high degree of protection against the toxic effects of *Treponema*

pallidum, they are no more capable of terminating the infection than is man.

Whether the same conditions hold true for recently isolated strains is not entirely certain, but since the latter exhibit the same tendencies to lymphoid involvement as the older strains, it is not unlikely that they too are capable of surviving in the rabbit for an indefinite period of time.

Once it has been shown that there is a constant and permanent localization of *Treponema pallidum* in the lymphoid tissues of infected animals, there are many applications of these facts which are quite obvious, and the possibility of utilizing them in connection with the preservation of stock strains and as a means of determining the presence of infection in experimental animals appeared to be of sufficient importance to warrant a special note upon this subject.

The method proposed for the preservation of stock strains of *Treponema pallidum*, when not in active use, is merely to keep a sufficient number of infected animals to guard against loss of the strain by their death. Serial transfers may be dispensed with. When it is desired to recover the organism for teaching or experimental purposes, a popliteal node may be excised with aseptic precautions, minced, and ground in a mortar; an emulsion is then prepared by the addition of about 1.5 cc. of sterile normal salt solution with further grinding. The resulting fluid is aspirated into a syringe fitted with a No. 22 gauge needle and about 0.5 cc. of the emulsion is injected into a testicle of one or more normal rabbits. In order to allow ample time for the development of a testicular infection, the inoculation should be made 6 to 8 weeks before the organism is needed.

Exactly the same method is applicable to the demonstration of infection in experimental animals. The circumstances should determine in each case when test inoculations are to be undertaken. In chemotherapeutic experiments, for example, it would appear to be advisable to follow the old system of clinical observation for at least 1 to 2 months before resorting to test inoculation; otherwise an infection which had been almost extinguished might not be given a sufficient opportunity to reestablish itself.

The essential requirements of the method are extremely simple: One is advised against complicating the technique by the use of

foreign substances to aid in the grinding of material; filtration is unnecessary; the injection of large amounts of fluid tends to produce inflammatory reactions in the testicles which may obscure subsequent lesions, and the use of strong antiseptics, either in the removal of lymph nodes or in making inoculations, is contraindicated.

Eberson and Engman (4) have used a similar method with success in demonstrating infection in the lymph nodes of human subjects with latent syphilis, thus establishing another analogy between the human and animal infection.

Positive results from testicular inoculation are usually not difficult to determine. It should be noted, however, that atrophy of the testicle may occur instead of the usual granulomatous enlargement and that in exceptional instances, infection may be recognized by the development of an adenopathy when no lesions can be detected at the site of inoculation.

By the use of the method described, a great saving in time and expense may be accomplished, and infection can be determined with comparative ease and with much greater certainty than was hitherto possible. However, until the delicacy of the method has been subjected to further test, negative results are still to be accepted with reserve.

SUMMARY.

Experiments carried out on rabbits infected with *Treponema pallidum* showed that there was a constant invasion and localization of the organisms in the superficial lymph nodes, that the infection persisted indefinitely, and that organisms could be recovered at any time from such nodes as the popliteals. Based upon these observations, a method is proposed for the preservation or recovery of stock strains of *Treponema pallidum* and for the demonstration of infection in rabbits.

BIBLIOGRAPHY.

1. Pearce, L., and Brown, W. H., *Proc. Soc. Exp. Biol. and Med.*, 1920, xvii, 164.
2. Brown, W. H., and Pearce, L., *Arch. Dermatol. and Syphilol.*, 1920, ii, 470.
3. Brown, W. H., and Pearce, L., *Am. J. Syphilis*, 1921, v, 1.
4. Eberson, F., and Engman, M. F., *J. Am. Med. Assn.*, 1921, lxxvi, 160.

STUDIES ON LYMPHOID ACTIVITY.

V. RELATION BETWEEN THE TIME AND EXTENT OF LYMPHOID STIMULATION INDUCED BY PHYSICAL AGENTS AND THE DEGREE OF RESISTANCE TO CANCER IN MICE.

BY JAMES B. MURPHY, M.D., WARO NAKAHARA, PH.D., AND ERNEST STURM.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Two methods have been described by which a definite stimulation of the circulating lymphocytes, accompanied by a hyperactivity of the lymphoid centers, may be brought about. It was first noted that small doses of x-rays would induce this condition,[1] but the reaction was of short duration[2] as compared with the stimulation occurring in cancer-immune mice following inoculation of cancer.[3] The stimulation induced by x-rays is preceded by a period during which evidences of the destructive action of this agent on the lymphoid centers are present, and is followed by a period during which the stimulation phase alone is present. By the 4th day the proliferative activity is at its height and then quickly subsides.

The amount of stimulus produced by dry heat[4] is much greater in extent and of longer duration than that seen after exposure to x-rays. Immediately after the heat application there are also marked evidences of cell destruction in the lymphoid centers, but the stimulation following is more prompt and of greater volume as judged by the blood pictures.

---

[1] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800. Thomas, M. M., Taylor, H. D., and Witherbee, W. D., *J. Exp. Med.*, 1919, xxix, 75.

[2] Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1920, xxxi, 13.

[3] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 204. Murphy, Jas. B., and Nakahara, W., *J. Exp. Med.*, 1920, xxxi, 1.

[4] Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1919, xxix, 1.

We have described the close relation existing between lymphoid stimulation and the resistant state to cancer inoculation. If this relation is quantitative in nature, it would be expected that mice whose lymphoid cells are stimulated by x-rays would show a definite degree of resistance but less than that exhibited by animals following heat stimulation. This fact is borne out by our figures on 102 mice heated 1 week before inoculation, which showed an average immunity of 60.3 per cent, controlled by 83 normal mice inoculated with the same tumors showing an average immunity of 16.5 per cent. On the other hand, 144 mice, x-rayed from 3 to 7 days before inoculation, had an immunity of 37.5 per cent; they were controlled by 137 normal mice inoculated with the same tumors, having an average immunity of 10.4 per cent.

If the hypothesis is true that stimulation of the lymphocytes of mice definitely reduces the number of takes of cancer grafts, it would be of importance to know whether there is a difference in resistance percentage when the tumor inoculation is made at varying times after the stimulus is administered. The following experiments were planned to test this point.[5]

Immunity after Exposure to X-Rays.

Mice of about the same age were divided into three lots. Two of these lots were given a dose of x-rays governed by the following factors: spark-gap $\frac{3}{8}$ inch between points, milliamperage 25, distance from target to back of animals 8 inches, and time 10 minutes. One lot received this dose 7 days and the other immediately before they were inoculated with a tumor (Bashford 63). At the same time the third group was inoculated with the same material and weekly measurements were made of the resultant tumors.

Table I gives the results of six experiments, the percentage of immune mice being estimated for a period 3 weeks after inoculation (Text-figs. 1 and 2).

[5] A preliminary report of this work was made before the National Academy of Science (Murphy, Jas. B., *Proc. Nat. Acad. Sc.*, 1920, vi, 35).

TABLE I.

Experiment No.	Group 1.		Group 2.		Group 3.	
1	11.1 per cent (9 mice).		0.0 per cent (10 mice).		0.0 per cent (10 mice).	
2	30.0 " " (10 ").		12.5 " " (8 ").		10.0 " " (10 ").	
3	10.0 " " (10 ").		0.0 " " (10 ").		0.0 " " (10 ").	
4	50.0 " " (10 ").		10.0 " " (10 ").		20.0 " " (10 ").	
5	40.0 " " (10 ").		11.1 " " (9 ").		11.1 " " (9 ").	
6	50.0 " " (10 ").		0.0 " " (10 ").		10.0 " " (10 ").	

Group 1 was made of mice given a dose of x-rays 7 days before cancer was inoculated. Group 2 animals were x-rayed immediately before the cancer inoculation. Group 3 comprised the control mice inoculated with the same cancers. 175 mice were used for these tests.
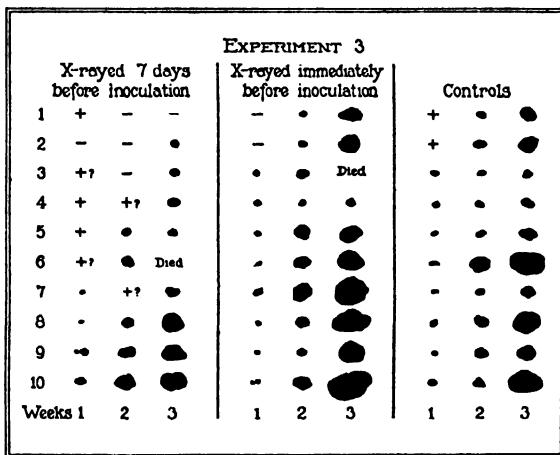
TEXT-FIG. 1. The effect of x-rays on the immunity to transplanted cancer when administered 7 days and immediately before inoculation.

From the foregoing experiments it is seen that the resistance of mice inoculated with cancer immediately after a stimulating dose of x-rays is no higher than that of normal mice, and on the average is somewhat lower. On the other hand, the mice inoculated with cancer a week after a stimulating dose of x-rays show a consistently higher degree of resistance which reveals itself both in the number of takes and in the rate of growth of the tumor.

TEXT-FIG. 2. The effect of x-rays on the immunity to transplanted cancer when administered 7 days and immediately before inoculation.

Immunity after Exposure to Dry Heat.

These experiments with x-rays have been paralleled with similar tests with heat as the agent for stimulating the lymphocytes.

Three groups of mice were used. Group 1 consisted of animals which had been heated 1 week previous to the inoculation with cancer. Group 2 mice were heated immediately before the cancer inoculation, and Group 3 consisted of the normal controls inoculated with cancer. The mice to be heated were placed in an enclosed cage 3 inches above an electric heating lamp. A thermometer was placed half an inch below the bottom of the cage. The temperature was allowed to rise to 55°C. before the animals were put in the cage and then they were left in for 5 minutes, the temperature rarely rising above 63°C. during this time.

The results of the three experiments are given in Table II, the figures representing the immunity percentage 3 weeks after inoculation with cancer (Text-fig. 3).

TABLE II.

Experiment No.	Group 1.			Group 2.			Group 3.		
7	66.6 per cent immunity.			40.0 per cent immunity.			20.0 per cent immunity.		
8	55.0 " " "			35.0 " " "			10.0 " " "		
9	50.0 " " "			30.0 " " "			10.0 " " "		

Group 1 was made up of mice heated 1 week before inoculation. Group 2 mice were heated immediately before inoculation, and Group 3 comprised the control mice. The results are based on 118 mice.

TEXT-FIG. 3. The effect of dry heat on the immunity to transplanted cancer when exposure to heat was done 7 days and immediately before inoculation.

The foregoing experiments indicate a definitely higher resistance in the mice inoculated immediately after heating than in the controls, while the mice heated a week prior to inoculation show an even more pronounced degree of immunity. The blood count after the heat treatment showed a sharp but very transitory drop in the number of circulating lymphocytes, followed within 24 hours by a marked increase and a continuous rise in these cells lasting for a week or more. Histological examination of the lymphoid organs of heated

animals showed that by 48 hours after the treatment these organs contain a larger number of mitotic figures in the germinal centers than is normally seen. The height of the reaction, judging from the blood pictures and the condition of the spleen and lymph nodes, occurs about the 7th day after the exposure to the heat. The degree of immunity to the transplanted cancer seems to vary directly with the amount of stimulation of the lymphocytes existing at the time of or immediately following the inoculation.

SUMMARY.

It has been shown that resistance to transplanted cancer follows stimulation of the lymphoid tissue when the stimulation is induced by either heat or small doses of x-rays. In this paper we have attempted to determine whether the degree of immunity had a quantitative relation to the amount of the stimulation. Fortunately, the two methods at our disposal give stimulation of markedly different characters. The small dose of x-rays gives a sluggish lymphoid cell reaction of short duration with a definite latent period between the treatment and the evidence of marked stimulation, while after heat a short period of depression is followed by a sharp stimulation continuing over a much longer period. The cancer inoculation into groups of mice made immediately after exposure to x-rays shows little resistance, while the inoculation made at the height of the stimulation phase shows a definite increase in the immunity. Animals inoculated with cancer immediately after the heat treatment exhibit a pronounced immunity, but not so marked as that shown when the inoculation is made at the height of the stimulation.

The amount of resistance shown when the cancer inoculation is made at the height of the moderately stimulating effect following exposure to x-rays, is much less than that seen when the inoculation is made at the height of the heat effect when the degree of stimulation is much greater. When the lymphocytosis sets in after the tumor graft is established only a slight effect is noted. All these results together are taken to indicate that the degree of immunity is dependent on the amount of lymphoid stimulation existing either at the time of or following soon after the cancer inoculation.

STUDIES ON X-RAY EFFECTS.

VII. Effect of Small Doses of X-Rays of Low Penetration on the Resistance of Mice to Transplanted Cancer.

By WARO NAKAHARA, Ph.D., and JAMES B. MURPHY, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

Murphy and Morton[1] have shown that small doses of x-rays capable of stimulating somewhat the circulating lymphocytes increase the resistance to the growth of spontaneous tumors in mice. In their experiments the tumor was first removed by operation, the animal was treated with one small dose of x-rays, and the graft of the original tumor was returned to the left groin of the same animal, any direct effect of x-rays on the tumor being thus avoided. Studies of the stimulative effect of x-rays, generated by a Coolidge tube, on lymphoid cells showed that the dose of x-rays governed by the following factors will increase lymphoid activity in the rabbit:[2] $\frac{7}{8}$ inch spark-gap, milliamperage 25, distance from target 8 inches, and time of exposure 20 minutes. This dose with its original factors was not satisfactory when applied to another species of animal, namely mice, but by shortening the time of exposure it was possible to induce an active proliferation of lymphoid tissue in these animals.[3] The best results were obtained when the exposure was of 10 minutes duration.[4]

There have been accumulated numerous facts which point to the conclusion that the activity of lymphoid cells is a factor governing resistance to the growth of cancer. With the determination of the x-ray dose which suffices to induce lymphoid stimulation, another opportunity was offered to test the part played by lymphoid cells in this state of resistance.

[1] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800.

[2] Thomas, M. M., Taylor, H. D., and Witherbee, W. D., *J. Exp. Med.*, 1919, xxix, 75. Nakahara, W., *J. Exp. Med.*, 1919, xxix, 83.

[3] Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1920, xxxi, 13.

[4] Murphy, Jas. B., *Proc. Nat. Acad. Sc.*, 1920, vi, 35.

EXPERIMENTAL.

Young adult white mice were exposed to the following dose of x-rays: spark-gap ⅛ inch, milliamperage 25, distance from target to back of mouse in the ordinary attitude 8 inches, and time of exposure 10 minutes. From 3 to 7 days afterwards the mice, together with a suitable number of controls, were inoculated subcutaneously in the left groin with a bit of a transplantable cancer (Bashford Adeno-carcinoma No. 63). The rate of growth of the tumor was noted at weekly intervals thereafter.

Table I gives the number of mice and the degree of resistance shown at the end of 3 weeks after inoculation (Text-figs. 1 to 3).

TABLE I.

Experiment No.	Interval between exposure to x-rays and tumor inoculation.	Immunity in x-rayed animals.	Immunity in controls.
	days		
1	3	40.0 per cent (10 mice).	11.1 per cent (9 mice).
2	5	50.0 " " (16 ").	10.0 " " (20 ").
3	5	20.0 " " (10 ").	0.0 " " (10 ").
4	7	75.0 " " (8 ").	23.3 " " (9 ").
5	7	10.0 " " (10 ").	0.0 " " (10 ").
6	7	30.0 " " (10 ").	0.0 " " (10 ").
7	7	71.4 " " (21 ").	40.0 " " (10 ").

During the course of these experiments the virulence of the tumor used varied considerably. In Experiments 3, 5, and 6 the controls showed no resistance, and correspondingly the immunity was low among the x-rayed animals. Attention is called particularly to Experiment 5 (Text-fig. 2) in which, at the end of 3 weeks, only one animal in the x-rayed series showed absolute immunity. A definite retarding effect was evident, however, in that at the end of the 1st week all but two of the controls showed definite tumors, while only three of the x-rayed animals proved to be susceptible. By the 2nd week after inoculation all the control mice had palpable tumors and only five of the x-rayed animals. This experiment is a very good illustration of how the more virulent tumors may break through a

resistance. While the degree of resistance induced by this dose of x-rays is low, yet it is consistently higher in the treated animals than in the controls, even with the varying growth energy of the tumors.
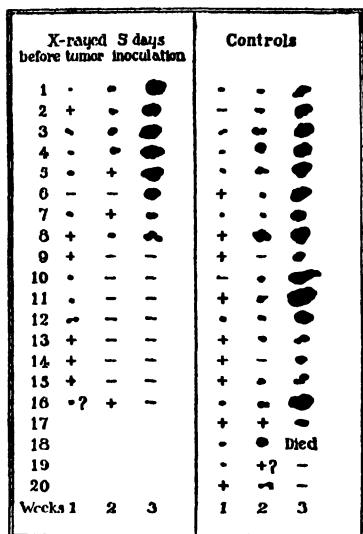
TEXT-FIG. 1.
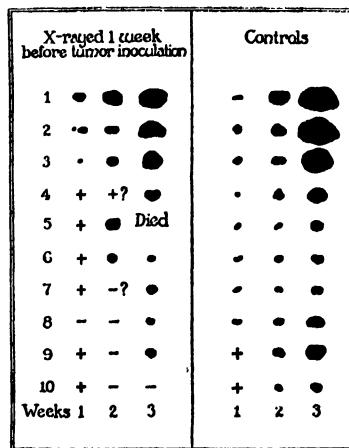
TEXT-FIG. 2.

TEXT-FIG. 1. Experiment 2. The rate of growth of Bashford Adenocarcinoma No. 63 in mice x-rayed before inoculation, contrasted with the rate of growth in untreated mice.

TEXT-FIG. 2. Experiment 5. The rate of growth of Bashford Adenocarcinoma No. 63 in mice x-rayed before inoculation, contrasted with the rate of growth in untreated mice.

DISCUSSION.

The foregoing experiments show conclusively that x-rays given in a dose sufficient to stimulate the lymphoid tissues, increase the resistance of mice to a transplanted cancer. As the degree of stimulation induced by this agent is less than that induced by exposure to intense dry heat or that following the injection of homologous living tissue, it is not surprising that the degree of resistance is less than that seen after the employment of the latter two methods.

It is of interest to note that the degree of resistance exhibited by the x-rayed mice varied with the growth energy of the tumor as shown by the number of takes in the control animals.

Our original observation that x-rays in small amounts increased the resistance of a mouse to transplants of tumors,[1] was later confirmed by Russ, Chambers, Scott, and Mottram,[5] who used transplants of the Jensen rat sarcoma with rats as their subjects. They gave an exposure of 12 seconds repeatedly, instead of the single dose which we have used.
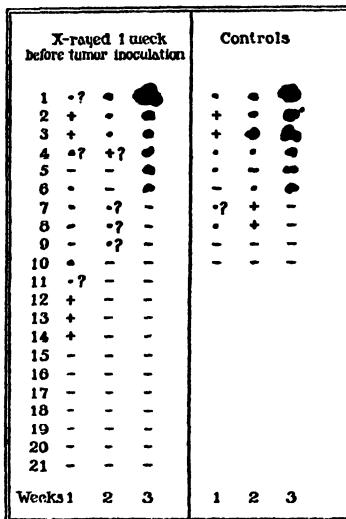
Text-Fig. 3. Experiment 7. The rate of growth of Bashford Adenocarcinoma No. 63 in mice x-rayed before inoculation, contrasted with the rate of growth in untreated mice.

SUMMARY.

A relatively increased degree of resistance in mice to a certain strain of transplantable cancer was demonstrated after treatment of animals with small doses of x-rays capable of stimulating lymphoid tissue. The refractory state induced was determinable 3 to 7 days after the dose of x-rays was given.

[5] Russ, S., Chambers, H., Scott, G. M., and Mottram, J. C., *Lancet*, 1919, i, 692.

STUDIES ON X-RAY EFFECTS.

VIII. INFLUENCE OF CANCER INOCULATION ON THE LYMPHOID STIMULATION INDUCED BY SMALL DOSES OF X-RAYS.

BY WARO NAKAHARA, PH.D., AND JAMES B. MURPHY, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

The studies carried out in this laboratory have shown that immunity to cancer, whether natural or induced, is attended by lymphoid stimulation.[1] X-rays have been employed to secure the stimulative effect on the lymphoid elements;[2] when of sufficient amount, they have been found to increase[3] the resistance of mice to cancer transplants[4] made at the height of the reaction. On the other hand, if the cancer inoculation is made immediately after the x-ray treatment, no unusual degree of resistance is exhibited.[5] Hence it would appear that the early inoculation of cancer must have in some way interfered with the development of the defensive mechanism. The present study has been planned to elucidate this point.

EXPERIMENTAL.

Experiment 1.—Twelve normal white mice were given the following dose of x-rays (Coolidge tube): spark-gap ⅛ inch, milliamperage 25, distance 8 inches, and time of exposure 10 minutes. The manner of applying the dose was the same as in experiments previously reported. As soon after the treatment as possible the animals were inoculated subcutaneously in the left groin with fragments of a Bashford adenocarcinoma, No. 63. Of these mice, six were killed for histo-

---

[1] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 204.

[2] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800.

[3] Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1920, xxxi, 13.

[4] Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1921, xxxiii, 429.

[5] Murphy, Jas. B., *Proc. Nat. Acad. Sc.*, 1920, vi, 35. Murphy, Jas. B., Nakahara, W., and Sturm, E., *J. Exp. Med.*, 1921, xxxiii, 423.

logical study 48 hours and the other six 4 days after the inoculation. No differ
ence was noted at autopsy between the two groups of mice killed at different
periods.

In three of the six animals in each group the spleen and lymph nodes were found
at autopsy to be smaller than is usual in normal mice. The mesenteric node,
which is the largest lymph node in the mouse, was in a few instances as small as
the normal inguinal, or even the axillary node.

Microscopically, a considerable number of pycnotic cells were found in these
organs, but the deposit of pigment in the spleen was never conspicuous. The
most striking feature was the almost complete suppression of the proliferative
activity of lymphoid cells in half of the animals examined, only a few mitotic
figures being found in each section. This suppressed activity was equally evi-
dent in the two groups, and apparently was independent of the size of lymphoid
organs. In the remaining half of the animals, mitotic figures were found more
frequently, but in no instance was there any sign of an activity above normal.

Experiment 2.—Eleven normal white mice were given the same dose of x-rays
as before; six were killed 48 hours and five 4 days after the treatment, without
having been inoculated with the cancer.[6]

The increase in the number of mitotic figures in the lymphoid tissue was evi-
dent in the majority of cases; namely, in nine out of eleven animals. The remain-
ing two animals showed only slight signs of lymphoid proliferation, judging by
the limited number of mitotic figures found.

Experiment 3.—Eleven normal white mice were treated with the same dose of
x-rays. 7 days later the animals were inoculated subcutaneously in the left
groin with fragments of Bashford Adenocarcinoma No. 63. Six of these mice
were killed after 48 hours and five after 4 days; *i.e.*, 9 and 11 days after the x-ray
treatment.

No unusual macroscopic feature of the lymphoid organs was noted at autopsy.
There was some variation in the histologic condition of these organs. In six of
the eleven animals, there was an extensive stimulation of lymphoid tissue as evi-
denced by the large number of mitotic figures in the tissue. The mitotic figures
were found abundantly, not only in the area of the germ centers, but also in the
pulp. The nodules were more or less enlarged. There were few pycnotic cells,
and pigment was almost entirely absent. In four other animals, the stimulative
reaction was similar in kind but somewhat less pronounced. One animal was
exceptional in that it showed almost no signs of cell proliferation.

Experiment 4.—Six normal white mice were treated with the same dose of
x-rays as those above but were not inoculated with cancer. Three were killed
9 days and three 11 days afterwards.

The spleen of four out of the six mice was found at autopsy to be abnormally
dark in color and, except in one animal in which it was below normal, of usual
size.

---

[6] This was a repetition, for the sake of comparison, of an experiment previously
reported.[3]

Histologically, the dark spleens showed a great accumulation of blood in the pulp, which was sparingly supplied with lymphoid cells. In contrast to the findings in Experiment 3, mitotic figures were in no instance abundant in the lymphoid tissue of spleen or of the lymph nodes. Cells with pycnotic nuclei were numerous, except in one specimen in which the number was small.

For a further comparison, the four preceding experiments were repeated in a single experiment which included groups subjected to the various experimental conditions of the individual experiments described above.

Experiment 5.—Twenty-five normal white mice, divided into four groups, were given the same dose of x-rays as in the previous experiments.

Group 1: eight mice were inoculated with Bashford Adenocarcinoma No. 63 immediately after the treatment with x-rays. They were killed 3 days later.

Group 2: seven mice were killed 3 days after the x-ray treatment without having been inoculated with cancer.

Group 3: six mice were inoculated, 7 days after the treatment with x-rays, with the same strain of cancer and were killed 3 days after the inoculation; *i.e.*, 10 days after the x-ray treatment.

Group 4: four mice uninoculated with cancer were killed 10 days after the x-ray treatment.

Of Group 1, all the mice except one individual showed suppression of the usual proliferation of the lymphoid elements. Of Group 2, all showed definite signs of increased proliferation of the same elements. In Group 3, three mice showed evidences of extensive stimulation, while the remaining three had a less marked reaction. Group 4 showed little signs of lymphoid proliferation.

Hence, the results of this experiment are in agreement with those of the four experiments immediately preceding.

The following conclusions can be deducted from the results. (1) Cancer inoculation made immediately after a stimulative dose of x-rays interferes with the lymphoid reaction and little or no stimulation results. (2) Cancer inoculation made at the height of the stimulation augments the lymphoid reaction, and the proliferative activity of the cells continues longer than in animals which have been stimulated but have not received the cancer inoculation. (3) A proportion of animals given the stimulative dose of x-rays fails to react. It is of considerable interest to ascertain whether mice which fail to react are susceptible to cancer inoculations. To determine this point we have used the blood counts to ascertain the absence or presence of a stimulative phase.
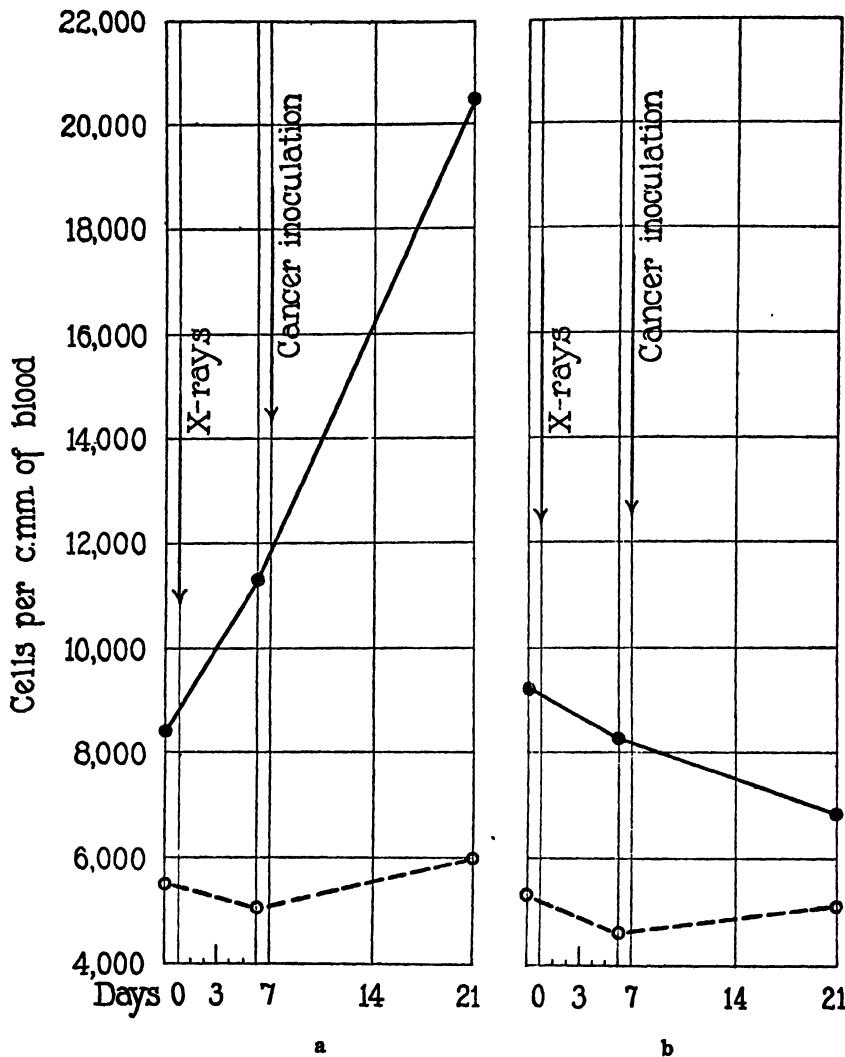
TEXT-FIG. 1, *a* and *b*. Experiment 6. Composite curves of white blood cell counts on mice x-rayed and inoculated with cancer 7 days later. (*a*) Composite curves from fourteen mice proved to be immune. (*b*) Composite curves from ten mice proved to be susceptible. ——————— Lymphocytes. ----------------- Polymorphonuclear leucocytes.

Experiment 6.—Blood counts were made on twenty-four mice which were then given an exposure of x-rays similar to that used in the preceding experiments. 7 days later a second count was made, followed by the inoculation of each animal with a graft of the Bashford mouse cancer, and 14 days later a third count. Fourteen of the twenty-four animals resisted and ten responded to the cancer inoculation.
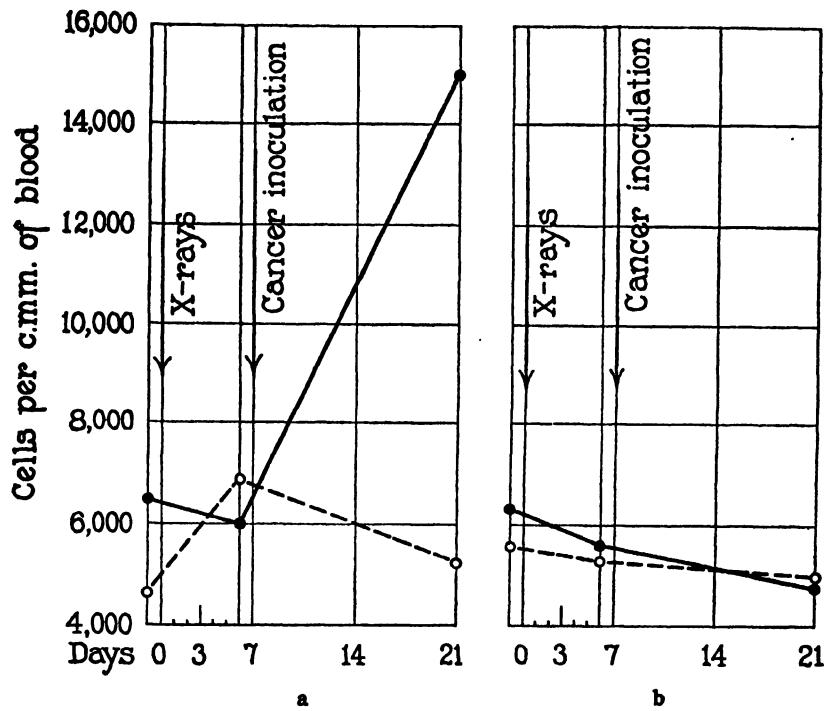
TEXT-FIG. 2, *a* and *b*. Experiment 7. Composite white blood cell counts on mice x-rayed and inoculated with cancer 7 days later. (*a*) Composite curves from the immune mice. (*b*) Composite curves from the susceptible mice. ——————— Lymphocytes. ------------------ Polymorphonuclear leucocytes.

In the immune mice (Text-fig. 1, *a*) the average number of lymphocytes per c. mm. of blood before x-ray treatment was about 8,300, the number of polymorphonuclear leucocytes being about 5,400. 6 days after the treatment (1 day before cancer inoculation) lymphocytes and polymorphonuclear cells were about 11,400 and 5,000 respectively. 2 weeks after the cancer inoculation the lymphocytes had, however, risen to approximately 20,000, while the polymorphonuclear cells showed but little change, being about 6,000.

In susceptible mice (Text-fig. 1, *b*) the average number of lymphocytes per c. mm. of blood before the dose of x-rays was approximately 9,600, the average number of polymorphonuclear cells was 5,300. A slight decrease in the white cells was noted 6 days after the treatment, the lymphocyte and polymorphonuclear leucocyte counts being 8,000 and 4,600 respectively. 2 weeks after the cancer inoculation the lymphocyte count was 7,000, the polymorphonuclear cell count 5,000.

Experiment 7.—The preceding experiment was repeated with twenty-six x-rayed mice, only nine of which resisted the cancer inoculation. Blood counts were made in the same way as before.

The average number of lymphocytes per c. mm. of blood in immune mice (Text-fig. 2, *a*) before x-ray treatment was about 6,500, and of polymorphonuclear leucocytes about 4,500. 6 days after the x-ray treatment the lymphocytes showed no material change, but the polymorphonuclear leucocytes went up to about 6,500. 2 weeks after the inoculation of cancer there was a rise of lymphocytes to about 15,000, while the polymorphonuclears decreased to about 5,000.

In susceptible mice (Text-fig. 2, *b*) the average number of lymphocytes and polymorphonuclear cells per c. mm. of blood before exposure to x-rays was about 6,000 and 5,000 respectively. No material change in these numbers was observed 6 days after x-rays. 2 weeks after cancer inoculation, however, the lymphocytes and polymorphonuclear cells were slightly decreased in number, the former being about 4,000 and the latter about 4,500.

DISCUSSION.

A comparison of the experiments described leads to the conclusion that if cancer inoculation is made immediately after the stimulative treatment with x-rays, no lymphoid stimulation occurs such as would regularly occur if the cancer inoculation were not made. On the other hand, if cancer inoculation is made 7 days after the x-rays are given, thus allowing the stimulation to develop before the inoculation, there is in the majority of cases what might be called a second stimulation of lymphoid tissue. It is significant, in connection with these facts, that while only little resistance to the transplantation was discovered when cancer was inoculated immediately after the x-rays, evidence of increased resistance appeared when inoculation was postponed until the 7th day.[5]

Attention is drawn in this connection to the parallelism existing between the lymphoid reaction accompanying the immunity to cancer grafts induced by physical agents (x-rays) and that induced by a biological agent (homologous blood). We have already shown

that mice immunized to cancer by means of an injection of defibri-nated blood show an increase in the number of mitotic figures in the lymphoid tissue. Such mice, when inoculated with a cancer graft 10 days after the injection exhibit a second stimulation of the tissue,[7] as well as a marked blood lymphocytosis.[1] The experiments reported indicate a corresponding effect brought about by small doses of x-rays. The blood counts on the animals after the cancer inocula-tion show that only the animals presenting an increase in the lympho-cytes prove to be resistant to the cancer. However, it should be stated that the blood counts were not made at a time to show the primary stimulative effect of x-rays, since this reaction, as previously shown, is of short duration. Hence it appears that as a result of the primary stimulation the animals have acquired the ability to react more strongly to a second stimulation; namely, the cancer inoculation.

SUMMARY.

Mice treated with small doses of x-rays and inoculated with cancer immediately afterwards, show a marked suppression of lymphoid proliferation. If, however, the cancer inoculation is made 7 days after the exposure to x-rays, thus permitting the primary lymphoid stimulation known to occur soon after the x-ray treatment to arise, a second stimulation takes place in a large proportion of mice thus inoculated.

Changes in the blood of mice x-rayed and inoculated with cancer 7 days afterwards show that the state of resistance to cancer inocu-lation is attended by blood lymphocytosis, as is the case in all other varieties of immunity to transplanted cancer so far studied.

[7] Murphy, Jas. B., and Nakahara, W., *J. Exp. Med.*, 1920, xxxi, 1.

EFFECT OF SMALL DOSES OF X-RAYS ON HYPERTROPHIED TONSILS AND OTHER LYMPHOID STRUCTURES OF THE NASOPHARYNX.*

BY JAMES B. MURPHY, M.D., WILLIAM D. WITHERBEE, M.D., STUART L. CRAIG, M.D., RAYMOND G. HUSSEY, M.D., AND ERNEST STURM.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 103 TO 108.

(Received for publication, March 11, 1921.)

In work carried on by us over several years we have extended the original observation of Heinicke[1] on the susceptibility of lymphoid tissue to x-rays and have shown other ways in which the x-rays may be employed as a therapeutic agent. It may be regarded now as established that the lymphoid tissue is more highly susceptible to x-rays than any of the structures of the body, except the sex glands, and that by suitable exposures it is possible to remove almost all of the lymphoid tissue without inducing detectable changes in other organs or tissues.[2] When the doses of x-rays are properly graded even the total number of polymorphonuclear leucocytes remains unaffected while the lymphocytes fall to a point at which few are seen in the circulating blood.[3]

The use of x-rays for reducing enlarged lymphoid organs is not new, but in the past the doses employed for the purpose have been large and thus have approached the danger point so closely that it has

---

* A preliminary report was made of this work before the American Society for Clinical Investigation and published in the Proceedings of the Society (Murphy, Jas. B., *J. Am. Med. Assn.*, 1920, lxxiv, 1738).

[1] Heinicke, H., *Mitt. Grenzgeb. Med. u. Chir.*, 1905, xiv, 21.

[2] Murphy, Jas. B., *J. Am. Med. Assn.*, 1914, lxii, 1459. Murphy, Jas. B., and Ellis, A. W. M., *J. Exp. Med.*, 1914, xx, 397.

[3] Taylor, H. D., Witherbee, W. D., and Murphy, Jas. B., *J. Exp. Med.*, 1919, xxix, 53.

been resorted to only in extreme instances. If, as our work indicates, extensive reduction of lymphoid tissue can be induced by small doses of x-rays, well within the safety limit, there is no reason why x-rays should not be used as a therapeutic agent in a variety of conditions. For obvious reasons the tonsil has been selected for the purpose of testing this point.

Histological examination of the tonsil shows it to have a structure similar to that of other lymphoid glands, with the exception of the fact that it is covered on one side by mucous membrane with crypts dipping down from the surface. These crypts have been described as natural test-tubes for the growth of bacteria. Whether or not the presence of pathogenic organisms in the crypts is the source of hypertrophy of the organ or whether the hypertrophy arises from another set of conditions is a moot point. It is, however, agreed that enlarged tonsils with resultant poorly drained crypts have a pathological significance.

In addition to the enlargement of the tonsil, other lymphoid deposits showing hypertrophy occur through the mucous membrane of the pillars of the fauces and as masses back of the posterior pillars. These structures also become pathologically altered in much the same way as the tonsil. They are not subject to surgical removal as is the tonsil but since they are made up of lymphoid cells are subject to influence by x-rays.

The following study was undertaken in order to test the effect of small doses of x-rays on the tonsil and other lymphoid deposits of the nasopharynx.

Technique.

The individuals to be treated are placed on a table with the head tilted so that the axis of the x-rays may pass under the angle of the jaw into the region of the tonsil. The area exposed on each side of the neck is about 3 inches square, the surrounding surface being covered with heavy sheet lead. The factors governing the dose of x-rays to each area were as follows: 8 inch spark-gap measured between points, 5 milliamperes, 10 inches distance from the target to the highest point of skin exposed; the time varies from 3 to 7 minutes, depending on the age of the individual, and the x-rays were filtered through 3 mm. of aluminum. The approximate value of this dose

is from one to one and three-quarters skin units. After an interval of a few weeks this treatment may if necessary be repeated with safety.[4] To insure immobility in young children a special board has been used with retaining straps and the child's head secured by means of a gauze bandage.

When excessive adenoid tissue was present a third area was exposed; namely, the back of the neck, just below the posterior occipital region with the head tilted forward. But this site of entry for the x-rays is less favorable, as will be indicated later in this paper.

Material.

This report is based on the study of 46 individuals ranging in age from $3\frac{1}{2}$ to 45 years and observed 1 month or longer after treatment. About 40 other individuals were treated, but as they did not return for examination they have not been included. The condition of the tonsils was noted in each individual and a drawing showing the size made by an artist independent of the examining physician. The state of the tonsils on first examination varied from that of simple hypertrophy to the enlarged organ with ragged surface and deep crypts containing exudate, or the small pathologically altered tonsil associated with symptoms of systemic disease. No individuals were treated at a time when the throat showed signs of acute inflammation.

The histories of eleven selected typical cases will be given and drawings of some of these, showing the progressive changes in the throat.

Case A.—J. L. L., white, male; age 19 years (Fig. 1).

Mar. 15, 1920. Throat: The tonsils, small and buried, the left larger than the right, ragged in appearance; numerous crypts containing exudate. Lymphoid

---

[4] In a later study Witherbee has recommended the use of fractional doses, four or more if necessary, treatments being given at 2 week intervals (Witherbee, W. D., *Am. J. Roentgenol.*, 1921, viii, 25). The factors of this dose are, 7 inch spark-gap measured between points, 5 milliamperes, 10 inches distance, time of exposure 3 minutes and 18 seconds; filtered through 3 mm. of aluminum. The principal advantage of this method is that it makes the treatment more flexible and the individual may be given more nearly the amount of x-rays necessary to induce the desired result.

tissue behind pillars increased in amount; large amount of adenoid tissue. Enlarged cervical lymph nodes. Bacteriological examination:[5] Right tonsil 50, left tonsil 150 colonies of hemolytic streptococci; vault none. X-rays: Three areas, right and left sides of neck and posterior occipital region, exposed to filtered x-rays; spark-gap 8 inches, milliamperes 5, time 6 minutes, filtered through 3 mm. of aluminum.

Mar. 22. Tonsil tissue markedly reduced and glazed and pale in appearance; lymphoid tissue behind pillars pale and smooth. Bacteriological examination: Right tonsil 50, left 150, and vault 50 colonies of hemolytic streptococci.

Apr. 5. Tonsil pale; edges of crypts inverted; no exudate can be pressed from tonsil. Adenoids considerably reduced; smooth and pale. Bacteriological examination: Right tonsil 100, left tonsil 50, and vault 150 colonies of hemolytic streptococci.

Apr. 26. Little visible tonsil tissue, of smooth appearance; no exudate; adenoid tissue small in amount, smooth and regular. Bacteriological examination: No hemolytic streptococci found.

Case B.—E. S., white, male; age 26 years (Fig. 2).

Dec. 10, 1919. Throat: Medium sized tonsils with deep crypts full of exudate; ragged inflamed surface. X-rays: Two areas, right and left side of neck, exposed to x-rays; spark-gap 8 inches, milliamperes 5, distance 10 inches, time 6 minutes, filtered through 3 mm. of aluminum.

Mar. 4, 1920. Tonsil shows marked shrinkage; some exudate present. X-rays: Two areas, right and left side of neck, exposed to x-rays in same dosage as above.

Apr. 4. Tonsils small; smooth surface; no exudate.

Mar. 5, 1921. Tonsils small with smooth surface; no exudate.

Case C.—H. W., white, male; age 14 years.

Mar. 17, 1920. Throat: Tonsils very large, buried; irregular surface; numerous crypts with yellowish exudate. Mass of lymphoid tissue back of pillars. Adenoid tissue, large mass covering surface of vault and fossa. Bacteriological examination: Right tonsil no colonies, left 50, and vault 50 colonies of hemolytic streptococci. X-rays: Three areas, right and left side of neck and posterior occipital region, exposed to x-rays; spark-gap 8 inches, milliamperes 5, distance 10 inches, time 6 minutes, filtered through 3 mm. of aluminum.

Mar. 24. Tonsils show decided reduction in size; pale, smooth surface; on pressure some whitish secretion. Adenoid tissue considerably reduced and of smooth, clean appearance. Bacteriological examination: Tonsils no colonies, vault 50 colonies of hemolytic streptococci.

Mar. 31. Tonsils show further reduction in size; smooth and pale; no exudate on pressure. Adenoid tissue pale, smooth, and clean; more normal in appearance. Bacteriological examination: Tonsils and vault show no hemolytic organisms.

Apr. 7. Improvement continues. Lymphoid tissue along posterior pillars has entirely disappeared.

---

[5] It is obvious that the number of colonies is roughly indicative only, as the quantity of material inoculated and part of organ touched with the loop are not subject to accurate control.

June 14. Tonsils flat; pale smooth surface; adjacent mucous membrane pale; edges of crypts inverted and crypts show retraction. Adenoid mass materially reduced. Bacteriological examination: No cultures taken.

Case D.—S. A., white, male; age 31 years.

Apr. 7, 1920. Throat: Tonsils moderately enlarged, buried; numerous crypts; purulent fluid on pressure. Marked hypertrophy of lymphoid tissue along posterior pillars. No adenoid tissue. Bacteriological examination: Right tonsil 200, left tonsil 200, and vault 200 colonies of hemolytic streptococci. X-rays: Three areas, right and left side of neck and posterior occipital region, exposed to x-rays. Spark-gap 8 inches, milliamperes 5, distance 10 inches, time 6 minutes, filtered through 3 mm. of aluminum.

Apr. 12. Tonsils show some reduction; pale and smooth; very little exudate on pressure. Marked reduction in lymphoid tissue on posterior pillars. Bacteriological examination: Right tonsil 150, left tonsil 200, and vault no colonies of hemolytic streptococci.

Apr. 19. Tonsils markedly reduced, smooth, pale, normal in appearance; less exudate on deep pressure. Further reduction in amount of lymphoid tissue on pillars. Bacteriological examination: Right tonsil 50 colonies, left tonsil no colonies, and vault 50 colonies of hemolytic streptococci.

May 10. Tonsils further reduced; mucous membrane of tonsil and pillars smooth and pale; edges of crypts rounded; small amount of exudate on deep pressure. Lymphoid deposit on pillars has practically disappeared. Bacteriological examination: No hemolytic streptococci found.

Case E.—J. V. K., white, male; age 15 years (Fig. 3).

Mar. 31, 1920. Throat: Tonsils large with ragged, irregular surface; numerous crypts with exudate. Large irregular mass of adenoid tissue. Bacteriological examination: Right tonsil 150 colonies of hemolytic staphylococci, 50 colonies of hemolytic streptococci; left tonsil 50 colonies of hemolytic streptococci; vault 100 colonies of hemolytic staphylococci. X-rays: Three areas, right and left side of neck and posterior occipital region, exposed to x-rays; spark-gap 8 inches, milliamperes 5, distance 10 inches, time 6 minutes, filtered through 3 mm. of aluminum.

Apr. 7. Tonsils slightly reduced; less ragged in appearance. Adenoids show some reduction. Vault contains mucopurulent discharge. Bacteriological examination: Right tonsil 100, left 50, and vault no colonies of hemolytic streptococci.

Apr. 14. Tonsils markedly reduced; surface irregular, ragged, and congested. Adenoid tissue reduced; smoother surface. Bacteriological examination: No hemolytic organisms found.

Apr. 21. Tonsils show still further reduction; surface smooth; edges of crypts inverted. Adenoid tissue markedly reduced; smooth pale surface. Bacteriological examination: No hemolytic organisms found.

Apr. 26. Tonsils flat; pale, smooth, clean surface; edges of crypts round and inverted. Bacteriological examination: No hemolytic organisms found.

May 24. Tonsils normal in appearance. Adenoid mass reduced in size.

Sept. 29. Tonsils very small; smooth, pale surface. Adenoids greatly reduced and normal in appearance.

Case F.—S. V. M., white, male; age 10 years.

Mar. 24, 1920. Throat: Tonsils large, partly buried; irregular, ragged surface; numerous crypts filled with thick yellow exudate. Lymphoid tissue behind pillars markedly hypertrophied. Large irregular mass of adenoid tissue covered with purulent exudate. Bacteriological examination: Right tonsil 200, left tonsil 100, and vault no colonies of hemolytic streptococci. X-rays: Three areas, right and left side of neck and posterior occipital region, exposed to x-rays; spark-gap 8 inches, milliamperes 5, distance 10 inches, time 6 minutes, filtered through 3 mm. of aluminum.

Mar. 31. Tonsils reduced in size; edges of crypts inverted; less exudate. Pharynx had dull red glazed appearance. Adenoid tissue considerably reduced; pale; less exudate. Bacteriological examination: Right tonsil 50 colonies of hemolytic streptococci, left tonsil and vault no hemolytic organisms.

Apr. 7. Tonsils markedly reduced; some surface secretion; crypts much cleaner. Adenoid tissue still further reduced. Bacteriological examination: No hemolytic organisms found.

Apr. 14. Tonsils very markedly reduced; surface smooth and clean; edges of crypts inverted and smooth; no exudate on deep pressure. Adenoid tissue smooth; normal appearance. Lymphoid tissue back of posterior pillars practically disappeared. Bacteriological examination: No hemolytic organisms found.

Sept. 29. Tonsils show further reduction; normal in appearance; edges of crypts smooth and inverted. Adenoid tissue still present; small amount of exudate on pressure.

Case G.—J. W., white, male; age 17 years (Fig. 4).

Mar. 24, 1920. Throat: Tonsils very large; ragged and congested. Considerable hypertrophy of lymphoid tissue behind posterior pillars. Bacteriological examination: Right tonsil 200, left tonsil 150, and vault 200 colonies of hemolytic streptococci. X-rays: Three areas, right and left side of neck and posterior occipital region, x-rayed; spark-gap 8 inches, milliamperes 5, distance 10 inches, time 6 minutes, filtered through 3 mm. of aluminum.

Mar. 31. Tonsils showed marked reduction; pale and smooth. Bacteriological examination: Right tonsil 100, left tonsil 50, vault 100 colonies of hemolytic streptococci.

Apr. 14. Tonsils show still further reduction; smooth, normal appearance. Lymphoid tissue behind posterior pillars entirely gone. Bacteriological examination: No hemolytic organisms found.

June 28. Tonsils small, smooth, pale; edges of crypts inverted and translucent.

Sept. 13. Tonsils show some further reduction; white bands around edges of inverted crypts; right tonsil shows some cheesy deposits, easily removed on pressure. Bacteriological examination: Right tonsil 50 colonies of hemolytic streptococci, left tonsil and vault no hemolytic organisms.

Feb. 17, 1921. Tonsils small, flat; surface smooth and pale; no exudate. Bacteriological examination: No hemolytic organisms found.

Case H.—J. Z., white, male; age 21 years (Fig. 5).

Mar. 10, 1920. Throat: Tonsils moderately enlarged; ragged surface; numerous crypts. Left tonsil has large fossa filled with purulent exudate. Moderate sized mass of adenoid tissue, irregular, covered with whitish secretion. Bacteriological examination: Right tonsil no hemolytic organisms; left tonsil 50 colonies of hemolytic streptococci and 200 colonies of hemolytic staphylococci; vault 50 colonies of hemolytic streptococci and 50 colonies of hemolytic staphylococci. X-rays: Three areas, right and left side of neck and posterior occipital region, exposed to x-rays; spark-gap 8 inches, milliamperes 5, distance 10 inches, time 6 minutes, filtered through 3 mm. of aluminum.

Mar. 17. Tonsils distinctly reduced; cleaner in appearance. Adenoids cleaner. Bacteriological examination: No hemolytic organisms found.

Mar. 31. Tonsils reduced; still some exudate. Adenoid tissue reduced, pale; less exudate.

Apr. 28. Tonsils show marked reduction; smooth, pale surface; crypts clean; no exudate on deep pressure.

June 14. Tonsils show still further reduction; no exudate from right tonsil, small amount from left on deep pressure. Adenoids possibly reduced.

Sept. 13. Tonsils small, smooth, pale; on deep pressure still some exudate; edges of crypts markedly inverted. Adenoid tissue still present.

Mar. 11, 1921. Tonsils small; normal in appearance.

Case I.—V. S., white, male; age 14 years (Fig. 6).

Mar. 3, 1920. Throat: Tonsils large, ragged; numerous crypts full of pus. Large mass of lymphoid tissue behind posterior pillars. Large mass of adenoid tissue. Bacteriological examination: Right tonsil 100 colonies of hemolytic streptococci and 50 colonies of hemolytic staphylococci; left tonsil 50 each of hemolytic streptococci and staphylococci; vault 50 colonies of hemolytic streptococci and 150 of hemolytic staphylococci. X-rays: Two areas, right and left side of neck, exposed to x-rays; spark-gap 8 inches, milliamperes 5, distance 10 inches, time 7 minutes, filtered through 3 mm. of aluminum.

Mar. 10. Tonsils reduced in size; smooth surface; less exudate on pressure. Lymphoid masses behind posterior pillars markedly reduced; smooth glazed appearance. Bacteriological examination: Right tonsil 50 colonies of hemolytic streptococci, left tonsil and vault no hemolytic organisms found.

Mar. 17. Tonsils still further reduced; pale, clean. Bacteriological examination: No hemolytic organisms found.

Mar. 24. Tonsils smaller; smooth surface. Further reduction in lymphoid tissue back of pillars. Bacteriological examination: No hemolytic organisms found.

Apr. 28. Tonsils show marked reduction in size; pale and smooth; no injection of mucous surfaces; no exudate on deep pressure; edges of crypts smooth. Lymphoid tissue behind posterior pillars practically disappeared. Adenoid mass reduced. Bacteriological examination: No hemolytic organisms found.

Sept. 13. Tonsils small and normal in appearance; no exudate on deep pressure; edges of crypts smooth and inverted; mucous surfaces show no injection. Lymphoid deposits back of pillars practically gone. Adenoids reduced but still large.

Case J.—E. C., white, male; age 16 years.

Mar. 22, 1920. Throat: Tonsils large; numerous crypts filled with yellowish exudate; large amount of cheesy material on pressure. Hypertrophy of lymphoid tissue on posterior pillars. Large irregular mass of adenoid tissue covered with yellowish exudate. Bacteriological examination: Right tonsil 200, left 150, and vault 150 colonies of hemolytic streptococci. X-rays: Two areas, right and left side of neck, exposed to x-rays; spark-gap 8 inches, milliamperes 5, distance 10 inches, time 6 minutes, filtered through 3 mm. of aluminum

Mar. 29. Tonsils considerably reduced; smooth and pale; no exudate on pressure. Adenoid tissue considerably reduced. Bacteriological examination: Right tonsil 200, left 150, and vault 150 colonies of hemolytic streptococci.

Apr. 5. Tonsils further reduced; pale and smooth; edges of crypts smooth; slight exudate on pressure. Adenoids markedly reduced; pale, smooth, and clean. Bacteriological examination: Right tonsil 50, left 50, and vault no colonies of hemolytic streptococci.

Apr. 14. Tonsils and adenoids markedly reduced; pale and smooth. Bacteriological examination: No hemolytic organisms found.

May 10. Tonsils small; normal in appearance; mucous surfaces pale; edges of crypts rounded and smooth; no exudate on deep pressure. Lymphoid tissue on posterior pillars has practically disappeared. Adenoid mass considerably reduced in size.

June 14. Tonsils normal in appearance. Adenoids very small, pale, smooth mass.

Sept. 29. Tonsils and adenoids small and normal in appearance; surface smooth; no exudate.

Case K.—J. F., white, male; age 16 years.

Mar. 8, 1920. Throat: Tonsils large, ragged; numerous crypts. Large mass of adenoid tissue. Enlargement of cervical glands. Bacteriological examination: Right tonsil 100, and left tonsil 50 colonies of hemolytic streptococci. X-rays: Two areas, right and left side of neck, exposed to x-rays; spark-gap 8 inches, milliamperes 5, distance 10 inches, time 7 minutes, filtered through 3 mm. of aluminum.

Mar. 15. Tonsils reduced; smooth, glazed surface. Adenoids considerably reduced; smooth and glazed. Bacteriological examination: Right tonsil 100, left tonsil 150, and vault no colonies of hemolytic streptococci.

Mar. 29. Tonsils further reduced; pale and smooth. Adenoids also reduced; pale and smooth. Bacteriological examination: No hemolytic organisms found.

Apr. 5. Right tonsil more reduced than left; surface pale and smooth. Adenoid mass pale, clean.

Apr. 12. Both tonsils further reduced; crypts inverted. Adenoid tissue considerably reduced. Bacteriological examination: Right tonsil 50 colonies of hemolytic streptococci, left tonsil and vault no hemolytic organisms.

May 10. Tonsils markedly reduced; surface normal; edges of crypts rounded and inverted; on deep pressure still slight amount of exudate. Lymphoid tissue behind posterior pillars completely disappeared. Adenoid tissue reduced but still large. Enlarged cervical glands somewhat smaller. Bacteriological examination: Right tonsil 5 colonies of hemolytic streptococci, left tonsil and vault no hemolytic organisms.

Sept. 29. Tonsils markedly reduced; normal in appearance; edges of crypts inverted, pale; no exudate. Adenoid tissue still present.

Table I shows the class of cases which have been treated with x-rays and the result of this treatment.

In general it may be said that the reduction in size of the tonsils and other lymphoid deposits in and behind the pillars of the fauces becomes clearly evident about 2 weeks after treatment and increases for 1 to 2 months. As the tonsil shrinks the crypts open and drain, and finally the edges become inverted and the surface grows smooth and healthy in appearance. The small lymphoid deposits in the pillars and the larger accumulation frequently present behind the posterior pillars of the fauces disappear promptly after the treatment and at the same time the injection of the vessels subsides.

The adenoid tissue is, as expected in view of the portal of entry used for the x-rays, not so uniformly reduced as the tonsils. This particular aspect of the problem of reducing excessive lymphoid tissue in the nasopharynx through x-rays is one to which in the near future especial attention will need to be given.

Bacteriological Examination.

Cultures were taken from the throats of 40 of the 46 individuals before and at intervals after treatment. The material for culturing was obtained from the crypts of the right and left tonsils, by means of a platinum wire about 3 inches long at the end of which was a small loop 2 mm. in diameter, bent at a right angle to the main piece of wire. The tongue was pressed down firmly with a tongue depressor, and the wire introduced into the crypt of the tonsil with a "stab and twist" motion. Caution was used to avoid contamination from

TABLE I.

Individual No.	Age.	Sex.	Tonsils.					Adenoids.	
			Size before treatment.	Condition.	Length of time under observation after treatment.	Size at last examination.	Condition after treatment.	Size and condition before treatment.	Size and condition after treatment.
	yrs.				mos.				
1	31	M.	+++*	Numerous crypts; mucopurulent exudate; considerable lymphoid tissue behind pillars.	2	++	Mucous membrane smooth; lymphoid tissue behind pillars almost gone; general appearance normal.	None.	
2	35	M.	+++	Soft, friable; numerous crypts; thin purulent exudate.	1	+	Edge of crypts smooth; normal.	Small.	Some reduction.
3	13	M.	++++	Ragged, inflamed; exudate.	6	++	Smooth, pale; right tonsil still not normal.	Large.	Little affected by treatment.
4	29	M.	++++	Ragged; deep crypts.	4	++	Crypts smooth and shallow; normal appearance.	Large.	Considerable reduction.
5	25	F.	++++	Not markedly abnormal.	3	++	Edges of crypt inverted; normal.	Small.	Reduction.
6	16	M.	++++	Numerous crypts; yellow, cheesy exudate; large mass of lymphoid tissue posterior to pillars.	6	++	Surface normal; no exudate in crypts; lymphoid masses posterior to pillars gone.	Large; covered with exudate.	Marked reduction; normal surface.

7	7	M.	+++++	Ragged; crypts contain mucopurulent exudate.	5	+++	Improvement; tonsils still not normal.	Large.	Reduction.
8	15	F.	+++++	Numerous crypts with purulent exudate.	1½ *wks.*	+++	Normal appearance; crypts inverted.	Large.	Reduction.
9	30	F.	+++	Ragged, inflamed; numerous crypts; exudate.	3 *mos.*	++	Clear, normal.	Medium.	Reduction.
10	?	M.	+++	Deep crypts with cheesy exudate; mass behind pillars.	5	++	Some exudate on deep pressure; surface normal; posterior pillar deposits absent.	Medium.	Reduction.
11	7	F.	+++	Ragged; deposits posterior to pillars.	5	++	Smooth; normal appearance; deposits posterior to pillars gone.	Large.	Marked reduction.
12	6½	F.	++++	Numerous crypts with ragged edges.	5	+	Normal.	Small.	No reduction.
13	15	M.	++++	Ragged, soft, congested.	1	++	Absolutely normal appearance.	Medium.	Marked reduction.
14	16	M.	++++	Numerous crypts; ragged edges; deposit behind pillars.	6½	++	Normal appearance; deposits behind pillars gone.	Large.	No marked reduction.
15	3	M.	++++	Ragged.	1	++	Much improved.	Very large.	Reduction.
16	6	F.	++++	Congested; numerous crypts; ragged; deposits posterior to pillars very large.	5½	++	Absolutely normal; deposit back of pillars gone.	Medium.	No marked reduction.

* ++++++ very much enlarged; +++++ much enlarged; ++++ very much enlarged; +++ medium enlargement; ++ approximately norm[al]
+ small; − not visible.

TABLE I—*Continued.*

Individual No.	Age.	Sex.	Tonsils.					Adenoids.	
			Size before treatment.	Condition.	Length of time under observation after treatment.	Size at last examination.	Condition after treatment.	Size and condition before treatment.	Size and condition after treatment.
	yrs.				mos.				
17	5	M.	++++	Ragged.	1½	+++	Edges of crypts inverted; smooth surface.	Very large.	No marked reduction.
18	49	M.	++++	Ragged; large deposit back of pillars.	6	++	Normal; posterior pillar deposits greatly reduced.	Large; ragged.	Some reduction; normal appearance.
19	28	F.	++++	Yellow, cheesy exudate; numerous crypts.	2½	+	Normal.	Medium.	Definite reduction.
20	28	F.	++	Ragged; numerous large crypts.	1	+	Normal; surface smoothed out.	Medium; mucopurulent exudate.	Reduction; no exudate.
21	15	M.	++++	Ragged; large crypts.	6	+	Normal; inversion of crypt.	Large; irregular.	Reduction; smooth.
22	35	M.	+++	Inflamed; ragged; numerous crypts; exudate.	1	++	Normal appearance; smooth, clean surface.		
23	7	F.	+++	Ragged.	2	+	Normal appearance.	Large, irregular.	Some reduction.
24	6	F.	++++	Ragged; numerous crypts; mucopurulent exudate.	2	++	Smooth, normal surface.		
25	26	M.	+++	Cheesy exudate.	3	+	Perfectly normal.	Small.	Reduction.

26	19	M.	+	Ragged; numerous crypts.	1	−	Normal appearance; crypts free from exudate.	Large.	Great reduction.
27	10	M.	++++	Ragged; purulent exudate in crypts.	6	+	Normal clean surface; crypts inverted.	Large.	Slight reduction.
28	7	M.	+++	Numerous crypts; pale and ragged.	2	++	Normal; crypts inverted.	Large.	No marked reduction.
29	33	M.	+++	Purulent exudate; crypts irregular with cheesy deposit.	10	+	Normal.	Medium.	Reduction.
30	6	F.	+++++	Irregular, ragged surface; mucous membrane injected.	2	++	Clean; mucous membrane pale.	Large.	No marked reduction.
31	4	F.	+++	Ragged, congested.	2	++	Surface clear.†	None.	Reduction.
32	17	M.	+++	Irregular surface, congested; thick yellow exudate; deposits posterior to pillars.	6	++	Surface pale and clean; normal.	Small.	
33	35	M.	+++	Yellow exudate in crypts; lymphoid tissue back of pillars.	1½	+	Normal appearance.		
34	45	F.	+++	Cheesy exudate.	5	+	Surface clear; one tonsil still has some exudate on deep pressure.		
35	31	M.	++++	Ragged, congested; purulent exudate and cheesy deposits.	1	++	Normal appearance.	None.	
36	14	M.	++++	Crypts contain pus; lymphoid deposits back of pillars.	6	+	Normal appearance; deposits back of pillars reduced.	Large.	Reduction.

† Later had an attack of tonsillitis.

TABLE I—*Concluded.*

Individual No.	Age.	Sex.	Tonsils					Adenoids	
			Size before treatment.	Condition.	Length of time under observation after treatment	Size at last examination.	Condition after treatment.	Size and condition before treatment.	Size and condition after treatment.
	yrs.				*mos.*				
37	36	F.	++	Mucopurulent exudate; lymphoid tissue back of pillars.	5	+	Normal.	Small.	No marked reduction.
38	17	M.	++++	Ragged, congested; lymphoid deposit back of pillars.	8	+	Smooth, pale; some exudate on deep pressure; lymphoid deposits gone.	None.	
39	21	F.	+++	Ragged, congested.	3	+	Normal.	None.	Little reduction.
40	30	F.	++++	Not abnormal.	1	+	Normal.	Small.	
41	23	F.	++	Mucopurulent exudate; cheesy deposits.	5	+	Smooth surface, normal appearance.	None.	
42	14	F.	++	Mucopurulent exudate; cheesy deposits; soft and friable.	5	+	Surface pale and normal; some exudate on deep pressure.	Large.	Little reduction.
43	14	M.	++++	Yellowish exudate.	3	+	Normal.	Large.	Reduction.
44	21	M.	++	Cheesy exudate.	1	+	Normal appearance; still some exudate on deep pressure.	Small.	Little reduction.
45	21	M.	++++	Subacute inflammatory condition.	8	+	Normal appearance; small amount of exudate on deep pressure.	Small.	Little reduction.
46	25	M.	+++	Inflamed; covered with mucopurulent exudate.	14	+	Absolutely normal.	None.	Little reduction.

the tonsil surfaces and the saliva. The material, so obtained, was placed on a blood agar plate, and gently spread over the surface with another platinum wire. The plates were then placed in the incubator at 37.5°C. Examinations of the plates and records of the findings were made after 24 and 48 hours of incubation.

Cultures were also taken from the nasopharyngeal vault by means of a very thin, semicircular, platinum wire, at the end of which was a small loop similar to that of the wire used in culturing the crypts of the tonsils. After firmly depressing the tongue, this wire was introduced into the vault directly back of the nose. The examination of the blood agar plates of these cultures was also made after incubating for 24 and 48 hours.

No attempt was made to differentiate the more common organisms usually found in the throat, such as pneumococcus, *Streptococcus viridans*, *Staphylococcus albus*, and *Staphylococcus aureus*. In distinguishing between hemolytic streptococcus and hemolytic staphylococcus, subcultures and stained films were resorted to. When the colony ranged in size from a pin-point to a small pin-head, and the area of hemolysis around the colony was from three to five times greater, streptococcus was usually found. Colonies which were as large as pin-heads or greater in diameter and about which the zone of hemolysis was very slight, appearing as a small halo, were usually staphylococcus.

36 of the 40 individuals showed hemolytic organisms to be present. Of these, seven became negative 1 week after treatment, fourteen after 2 weeks, eight after 3 weeks, and one after 4 weeks, making a total of 30 of the 36 treated cases which became negative. The six which continued to show hemolytic organisms were lost track of before further observations could be made.

Blood Counts.

The results of the x-ray treatment on the white blood cell count were not uniform. The counts were made at irregular intervals after meals and after a walk of several city blocks. When the lymphocytes were reduced in numbers the reduction was slight and of short duration, which would indicate that the systemic effect of the dose used was very slight.

DISCUSSION.

The small series of cases reported here shows the possibility of materially reducing the lymphoid deposits of the nasopharynx by comparatively small doses of x-rays. Animal experiments had shown that it is possible with x-rays to induce any degree of atrophy of the lymphoid tissue without damaging other tissues. In the series of treated individuals, in all but three or four instances one treatment gave an entirely satisfactory result. In two refractory cases a second treatment was followed by the desired degree of atrophy and a clearing up of the pathological condition. It is most probable that the other few individuals who did not respond to the one treatment would have yielded on further exposure to x-rays but unfortunately the observations were discontinued before this point could be determined.

The degree of atrophy to be aimed at is a matter that experience will decide. If a reduction below the normal size and the clearing up of obvious pathological states is sufficient as has been indicated in the majority of the cases treated and observed by us, there seems to be no reason for carrying the treatment beyond this point. In view, however, of the mild nature of the treatment recommended it appears entirely safe to repeat it at suitable intervals so as to secure almost any degree of atrophy that may be desired.

Our original idea in taking up this work was that the excess of lymphoid tissue interfered with the clearing up of local infections of the pharynx. It seems probable, however, that the disappearance of infection of the tonsils and change in bacteriological flora after x-ray treatment are due to the opening up and proper drainage of the crypts which follow atrophy, rather than the actual removal of the excess lymphoid tissue.

Tonsils which have been exposed to the x-rays and not sufficiently reduced in size would in all probability be as amenable to surgical removal as before the x-ray treatment, for we have never seen any evidence of fibrosis in the lymphoid organs of animals after similar treatment. The fibroid tonsil would probably not be reduced materially by x-rays, as fibrous tissue is not appreciably affected by this agent.

SUMMARY.

46 individuals with tonsils both hypertrophied and otherwise pathologically altered and some of whom had in addition adenoid masses and lymphoid deposits posterior to the pillars of the fauces, were given exposures to x-rays. In all but four cases the treatment was followed by marked atrophy of the tonsils and the other lymphoid deposits, attended by an opening and drainage of the tonsillar crypts. As this process progressed the previously enlarged tonsils assumed a smooth and normal appearance and the hemolytic bacteria—streptococcci and staphylococci chiefly—which were often present in the affected tonsil disappeared usually within 4 weeks of the treatment.

EXPLANATION OF PLATES

PLATE 103.

FIG. 1, *a* to *d*. (*a*) Condition of the tonsils before treatment; small and partly buried, with ragged surface and crypts containing exudate. In addition there were deposits behind the posterior pillars. (*b*) 1 week after x-ray treatment, showing reduction, and smoothing out of surface. (*c*) 3 weeks after treatment, with further reduction; no exudate could be expressed from tonsils. (*d*) 6 weeks after treatment. Tonsils not visible till anterior pillar was pulled back; normal in appearance. Hemolytic streptococci were present at the first examination but had disappeared by the 6th week after treatment.

PLATE 104.

FIG. 2, *a* to *f*. (*a*) The tonsils before treatment; medium size, with deep crypts containing exudate; ragged, inflamed surface. (*b*) 8 days after treatment; some reduction. (*c*) 26 days after treatment; tonsils congested and exudate still present. (*d*) 55 days after treatment; tonsils reduced but not normal. (*e*) 1 week after a second x-ray treatment, showing further reduction, and smoothing out of surface. (*f*) 1 year and 1 month after second treatment; tonsils small and normal in appearance.

PLATE 105.

FIG. 3, *a* to *d*. (*a*) Condition of tonsils before treatment; ragged, irregular surface, with crypts containing exudate. (*b*) 2 weeks after x-ray treatment; tonsils markedly reduced; surface irregular and congested. (*c*) 7 weeks and 5 days after treatment; tonsils normal in appearance. (*d*) 6 months after treatment; tonsils very small and normal. Hemolytic streptococci, present on first examination, disappeared after treatment.

PLATE 106.

FIG. 4, *a* to *e*. (*a*) Tonsils before treatment; very large, ragged, and congested. Hypertrophy of lymphoid tissue behind posterior pillars. (*b*) 3 weeks after treatment; tonsils reduced in size. Lymphoid tissue behind posterior pillars entirely atrophied. (*c*) 2 months after treatment; tonsils further reduced; normal in appearance. (*d*) About 6 months after treatment; tonsils further reduced; normal surface; still some exudate on deep pressure. (*e*) 11 months after treatment; tonsils small, flat; no exudate. Hemolytic streptococci disappeared from the throat after treatment.

PLATE 107.

FIG. 5, *a* to *e*. (*a*) Condition of tonsils before x-ray treatment; enlarged; ragged surface; deep crypts, with purulent exudate. (*b*) 7 weeks after treatment; tonsils markedly reduced; smooth, pale; no exudate on deep pressure. (*c*) 14 weeks after treatment; tonsils still further reduced; surface normal; no exudate. (*d*) 6 months after treatment; tonsils small and normal. (*e*) 1 year and 1 month after treatment; tonsils small and normal. Hemolytic streptococci disappeared from the throat by 1st week after treatment.

PLATE 108.

FIG. 6, *a* to *e*. (*a*) Tonsils before x-ray treatment; large, ragged; crypts contain pus. Large mass of lymphoid tissue behind posterior pillars. (*b*) 2 weeks after treatment; tonsils reduced; surface smooth and clean. Mass behind pillars reduced. (*c*) 4 weeks after treatment; tonsils markedly reduced; pale and smooth; no exudate on deep pressure. (*d*) 8 weeks after treatment; small amount of exudate. (*e*) 6 months after treatment; tonsils small, normal in appearance; no exudate on deep pressure. Lymphoid tissue behind pillars practically gone. Hemolytic streptococci disappeared from throat by 2nd week after treatment.
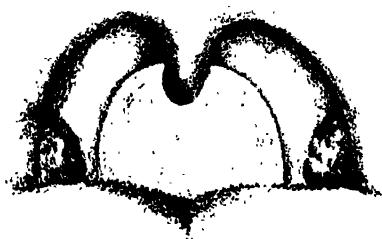
a
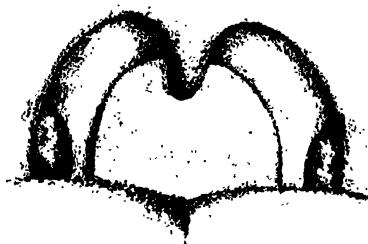
b

c

d
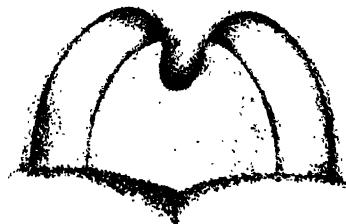
FIG. 1.

a
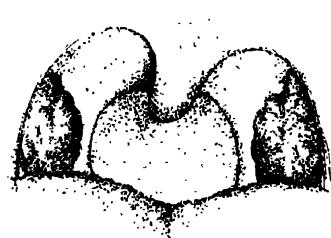
b
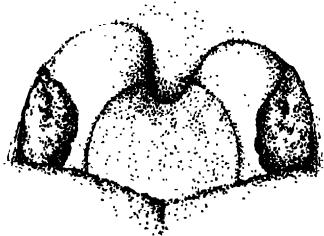
c
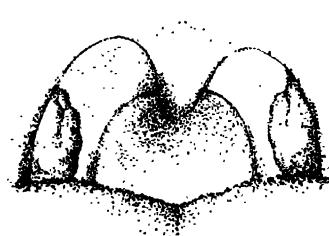
d
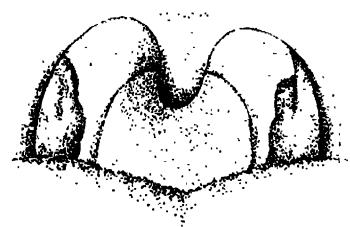
e
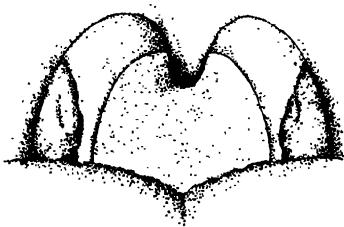
f

FIG. 2.

(Murphy, Witherbee, Craig, Hussey, and Sturm: Hypertrophied tonsils.)

FIG. 3.

a

b

c

d

e

FIG. 4.

(Murphy, Witherbee, Craig, Hussey, and Sturm: Hypertrophied tonsils.)
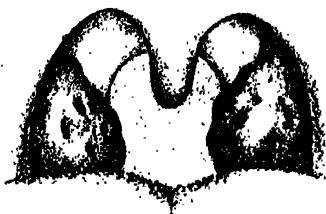
a
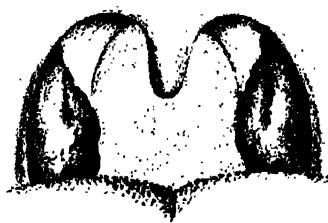
b

c
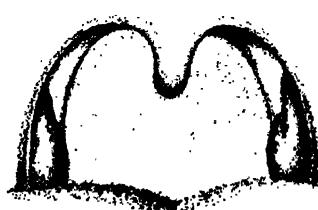
d
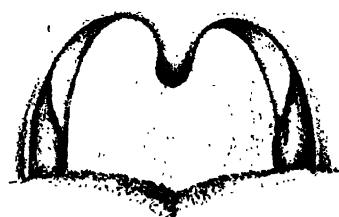
e

FIG. 5.

(Murphy, Witherbee, Craig, Hussey, and Sturm: Hypertrophied tonsils.)
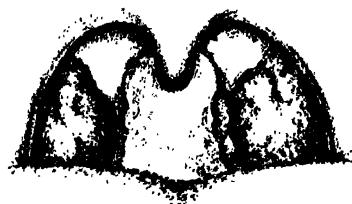
FIG. 6.

(Murphy, Witherbee, Craig, Hussey, and Sturm: Hypertrophied tonsils.)

ON THE DURATION OF CONSTRICTION OF BLOOD-VESSELS BY EPINEPHRIN.*

BY S. J. MELTZER AND JOHN AUER.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

INTRODUCTION.

In the history of physiology of blood pressure the discovery of vaso-constrictor and vasodilator nerves in the fifties of the last century by the classic physiologists Claude Bernard, Brown-Sequard and Schiff, marked an important event. It was made by simply observing the behavior of the blood vessels in the ears of rabbits after section of the sympathetic nerve, and after stimulating the central end of the sectioned nerve. In the desire to obtain quantitative data and preservable illustrative records, bloodpressure was studied in physiology in the subsequent years chiefly by the graphic method. When later another important event in the physiology of bloodpressure was brought to light namely, the action of the suprarenal extract upon the bloodvessels, it was studied nearly exclusively by the graphic method. It is the method of choice to this day. It is true that Oliver and Schafer (1) in their elaborate paper state that "direct ocular evidence upon both the large and the small blood-

---

* The nomenclature in the literature on adrenal extract is confusing. The following explanation of the terms we use in this paper may not be amiss. Three conditions are to be distinguished. 1. The secretion of the adrenal glands, or its principle as it exists in the living body, or is supposed to exist there. For this we accept the term adrenin, suggested by Schafer and being used by various authors abroad and in this country. 2. The active principle as isolated outside of the body (adrenalin, suprarenin, etc.). Abel was the first to isolate the principle in the form of a mono-benzoyl derivative and named it epinephrin. The American Pharmacopeia made this designation official. We shall use it as a generic name. 3. The particular preparation which is used in a specific investigation. In this series of experiments the adrenalin solution of Parke, Davis and Company was used.

vessels" was one of the methods employed in their studies. This was apparently done by simply looking at the bloodvessels in the tissues after a surgical exposure. But the exposure of normally protected bloodvessels to atmospheric air is a circumstance which can not well be disregarded when estimating ephemeral changes in the size of these vessels.

As far as we know, S. J. and C. Meltzer were the first to study the action of epinephrin upon bloodvessels by ocular observation of the rabbits' ear. While trying to establish the share which the sympathetic and the large auricular nerves possess in the innervation of the ear vessels (2) they discovered that the reaction of these vessels to an intravenous injection of adrenalin differs in ears deprived of their vasomotor innervation from that of normal ears in several respects: the latent period is longer, the development to the maximum and the decrease to normal is slower, and the entire duration of blanching of the denervated part is much longer than that of the normal ear (3). Later these authors administered adrenalin to rabbits by subcutaneous injection in some part of the back. They found that large doses which are capable of causing a prostration of the normal animal produce a blanching of the ears. However, subcutaneous adminstration of medium or rather small doses, while affecting the denervated ear in the same direction as an intravenous injection, causes rather a dilatation of the vessels of the normal ear. (4). Finally in studying the influence of the sympathetic nerve and the superior cervical ganglion upon inflammation in the ears of rabbits, these authors (5), in order to bring out certain differences, injected adrenalin subcutaneously in the ear itself; it caused a contraction of the dilated vessels in the "inflammatory area" (Entzündungshof) but did not affect the vessels in the "inflammatory focus" (Entzündungsherd).

The study of the action of epinephrin upon bloodvessels in the rabbits' ear is in many respects an advantageous method. The animal remains in a normal state; it is not exposed to the influence of an anesthetic and need not undergo preliminary operations. No apparatus is required for the observation. By this method medium-sized arteries and arterioles, veins and even capillaries (paleness and

flushing of the areas between visible vessels) are studied with ease. By this method the bloodvessels are studied directly and not the average bloodpressure of the entire animal; they are studied in an organ, the ear normally connected with the circulation of a living animal and not in a perfused organ, which is provided only with nervous and circulatory mechanisms and not, in addition, with mechanisms for chemical activities which might modify the action of adrenin upon the bloodvessels.

In two studies the present writers (6) established the unmistakable difference in the results of intramuscular and subcutaneous injections. The effect of an intramuscular injection sets in practically as rapidly as after an intravenous injection, while the effect of a subcutaneous injection upon bloodpressure develops slowly and is comparatively insignificant. In giving a subcutaneous injection, great care must be taken that the needle does not enter muscles or pass beneath a muscle fascia; such a mishap is not likely to occur when subcutaneous injections are given in the ear; the muscles of that structure are small and thin, and it is hardly possible to make there an "intramuscular" injection.

EXPERIMENTAL.

During the last few years we carried out experiments at various times in which adrenalin was injected subcutaneously in one ear of a rabbit and the course of the reaction of the various bloodvessels was studied by ocular observation. We wish to report here the results obtained in these experiments. In the following account, one or two protocols will be given to illustrate the several groups of experiments. Many of these accounts will be very brief and will consist essentially in a summary.

In the present series of experiments mostly the commercial solution of adrenalin chlorid of Parke, Davis and Company (1:1000) was used. In a few instances the solution was warmed for the purpose of removing the chloretone. It was shown later, however, that the warming could be dispensed with. Injections of chloretone, or of chloretone with HCl of an acidity equal to that of the adrenalin chlorid, had no effect upon the conditions of the blood vessels. In a few experiments the solution was freshly prepared in the laboratory from adrenalin crystals.

In the first few experiments the injection of adrenalin was made subcutaneously on the external surface of the tip of the right ear near the central artery and below its inner branch.

Experiment 1.—White female rabbit, 1440 grams. 2.35 p.m. Injected 0.3 cc. adrenalin; lost one drop. Observed until 5.05 when the right ear was still pale. Next day the right ear flushed definitely more than the left.

Summary.—There was a strong constriction of practically all the bloodvessels of the injected ear. There was no flushing phase after handling of the animal. The palor was uniform and lasted about three hours; (no observations made after this time). Only the outer lower third (external to the central artery) showed one hundred minutes after the injection a slight dilatation of the vessels. The left, uninjected ear became engorged shortly after the injection into the other ear. But after five minutes the ear vessels of the left ear showed quite rapid fluctuations. Next day the right, injected ear gorged more readily than the left, and stimuli which caused blanching of the left ear did not affect the engorgement of the right ear.

Experiment 2.—White female rabbit, 2330 grams. 11.52 a.m. Injected 0.3 cc. adrenalin; (about five drops lost). Observed until 5.05 when injected ear was still fairly pale.

Summary.—In this experiment 0.3 cc. was injected and about five drops were lost, that means that actually only a very small amount of adrenalin was injected. Nevertheless there was no dilatation of the vessels in the injected ear. On the contrary, the constricting effect set in immediately after injection and lasted several hours. The intensity of the constriction, however, was less than in the foregoing experiment. The uninjected ear paled for a very brief time and was followed by an engorgement.

The two foregoing experiments showed unmistakably that an injection of a small quantity of an adrenalin solution in the tip of the ear causes at once a paleness of practically the entire ear which lasts for several hours. The question arose: Is the effect due to adrenalin? In the tip of the ear the connective tissue is rather dense. A subcutaneous injection of 0.3 cc. of any solution will cause in this place considerable pressure. Could not the observed effect be due to the mechanical factor of pressure and not to the chemical element of epinephrin? This point was tested in experiments with saline of which the following is an example.

Experiment 3.—White female rabbit, 1500 grams. 10.17 a.m. Injected 0.3 sterile 0.9 per cent NaCl subcutaneously into the tip of the right ear.

Summary.—After an injection of 0.3 cc. of 0.9 per cent NaCl into the tip, two thirds of that ear became pale at once and the central artery became small. How-

ever, the paleness began to recede eight minutes later, the middle third being already pink and the central artery beginning to get wider. Twenty-three minutes after injection there was paleness only in the upper fifth; in the other part this ear resembled the non-injected ear. Immediately after the injection a ridge appeared along the central artery extending to the lower third. That ridge had largely disappeared in less than thirty minutes. It seemed to have been formed by the presence of fluid within the sheath of the artery. The injection in the one ear did not seem to have any effect upon the other ear.

Experiment 3 shows that an injection of normal saline into the tip of the ear causes indeed paleness if a large part of the ear and the central artery becomes narrow. The paleness however begins to disappear in less than ten minutes and is practically gone in less than thirty minutes. In the two experiments in which adrenalin was injected, the paleness persisted uniformly for more than one hour and a half and remained greatly in evidence for more than two hours, or rather as long as it was observed. While we have to admit that the pressure caused by the injection of adrenalin may be an important element in the early and sudden development of the blanching of the ear, there can be no doubt that it is the chemical property of the injected solution which is largely responsible for the long duration of the blanching.

In order to eliminate the mechanical factor as much as possible, the injections were made henceforth in the base of the external surface of the ear, where the subcutaneous tissue is more loose than in any other part of the ear. The pressure caused by the subcutaneous injection of fluid in this place could hardly drive it upwards mechanically; it is more liable to force the fluid into the loose tissues of the neck and to the tissues of the base of the other ear. The needle was introduced just above the transverse vein, pushed downward under the vein and the fluid injected below it.

Experiment 4.—White female rabbit, 1740 grams. 2.48 p.m. Injected 0.5 cc. adrenalin subcutaneously into the base of the right ear, externally to the central artery and about midway between it and the outer margin of the ear. Observed until 5.05 the same day and the next day after 10.30 a.m.

Summary.—The injection of 0.5 cc. of adrenalin into the base of the ear caused paleness of the injected ear. But the intensity of the pallor was not as great as after an injection in the tip. The pallor did not set in at once; it developed gradually. The injection was made externally to the central artery and the pallor was

greater in this part. (The central vessels adhere to the skin and form a sort of a partition between the outer covering and the cartilage of the ear) The paleness lasted for nearly three hours, that is, as long as it was observed. Next day it was the injected ear which showed a distinct tendency to dilatation.

The non-injected ear showed promptly a tremendous dilatation shortly after injection.

Experiment 5.—(Full protocol.) White female rabbit, 1760 grams. 11.08 a.m. Injected 0.5 cc. adrenalin subcutaneously into the base of the left ear; 0.25 cc. on both sides of the central artery, near the artery.

11.08½ Left ear pale, right more full; marked difference; right ear fluctuates; left pale from the base to the tip.

11.10 Very marked difference; left pale, no blood in central artery and upper branches. Right fills up and then pales on excitation.

11.30 Left very pale; artery not visible; veins very small and narrow. Right dilates only moderately.

12.15 Left very pale; artery practically not visible; veins very small; ear slightly bluish. Right fills up only moderately.

12.33, 2.00 and 3.00 p.m. Same as at 12.15 p.m.

4.25 Left central artery shows as a thin line now; left ear still considerably paler than right.

Next day 10.30 a.m. Both pale at first, then left flushes well, followed by right; both finally equal (left perhaps a bit wider.)

Summary.—In experiment 5, 0.5 cc. adrenalin was injected near the artery, 0.25 cc. on each side. In thirty seconds the left ear became pale from base to tip and soon there was no blood in central artery and its branches. This extreme blanching maintained for four hours, and even after nearly five and a half hours the constricting effect was still in evidence. Next day there was some tendency of the vessels of the injected ear to dilatation.

Experiment 6.—(Full protocol.) White male rabbit, 1600 grams. 1.33 p.m. Injected 0.3 cc. adrenalin each side of the central artery of the left ear, away from central artery, near outer and inner margins. For twenty minutes no effect can be seen; left ear cooler than right; then smaller arteries of the left ear paled.

2.20 Now left central artery moderately contracted, left ear cooler.

2.40 Left central artery now entirely empty.

2.50 Left central artery relaxed moderately.

3.05 Left ear pale, right moderately gorged; left central artery shows occasionally small sections of blood passing slowly upwards.

4.50 Left ear pale, central artery not visible; right ear after excitation now as pale as left. But shortly right ear gorges while left remains pale.

Next day 10 a.m. Both pale at first, left perhaps a trifle less than right. After some time both filled up strongly and equally, later maximal engorgement, equal.

Summary.—In experiment 6 the injections were made, 0.3 cc., near each margin away from the central artery. For twenty minutes there was no visible effect although the ear began to be cooler; the smaller arteries began to constrict at

least twenty minutes before the central artery was seen to be moderately contracted. Gradually the blanching became well pronounced and lasted for about three hours after the injection. Spreading from the tissue of the margin seems to be a slow process and it appeared to reach some distant small vessels before it reaches the central artery.

In the above experiments with injection of adrenalin into the base of the ear there was a definite, long lasting blanching of the ear and a constriction of the central artery. In experiments in which the injected adrenalin came immediately in contact with the central artery, the blanching set in practically at once, which seemed to be initiated by the constriction of the central artery. When the adrenalin was injected about midway between the margin and the central artery, the blanching and the constriction of the vessels developed gradually, and in the experiment in which 0.3 cc. of the adrenalin was given near the ear margins, the blanching began to make its appearance about twenty minutes after the injection, although the lower temperature of the injected ear was manifest before. The constriction of the central artery became evident much later than that of the smaller vessels. In outspoken cases of blanching there was no doubt that the veins participated in the constriction; but now and then some veins contained dark blood. When the constriction was not maximum, the central artery often had a moniliform appearance and short narrow sections were seen to be traveling in a peripheral direction. The constricting effect lasted in some instances as long as six hours, and certainly never less than three hours. In most of the experiments, even with intact innervation, a tendency to dilatation in the injected ear was manifest the day after the injection. In several experiments the blanching of the injected ear was accompanied by an engorgement of the other ear, the flushing setting in shortly after the injection.

Considering the readiness with which fluid injected into the loose tissue of the base of the ear makes its escape into the loose tissue of the neck, a quantity of 0.5 cc. may amount to a very small dose, especially when compared with a dose of 0.3 cc. injected into the very dense tissue of the tip of the ear. In the following experiments larger quantities were tested.

Experiment 7.—White female rabbit, 1920 grams.   10.59 a.m.   Injected 1 cc. adrenalin subcutaneously into the base of the left ear below the transverse vein, midway between the central artery and the external margin of the ear.

11.03   No definite difference between ears.

11.10   Both fairly pale on handling; then right ear fills up quite promptly, while left ear vessels dilate only very slightly.   Observed until 4.27 p.m. the same day and again at 10 a.m. the next day.

Summary.—In experiment 7, 1 cc. of adrenalin caused a markedly greater effect than 0.5 cc. when injected in a similar manner.   The development was slow, but the constriction and paleness attained a marked degree and lasted for more than five hours.   There was only a suggestion of a dilating effect upon the other ear and there were no definite after-effects the day following the injection.

Experiment 8.—(Full protocol.)   White female rabbit, 1600 grams.   11.34 a.m. Injected 0.8 cc. adrenalin into the subcutaneous tissue at the base of the left ear, 0.4 cc. each side of the central artery, and near it.

11.35   Both ears flush strongly, then left central artery begins to pale from below upward until only a thin red line remains; rest of left ear (veins) well distended.   Right ear flushed; central artery wide.

11.36   Left ear pale, veins emptied; right slightly pale but central artery well dilated.

11.40   Left pale, only little blood in veins; artery a thin red line.   Right shows a fairly dilated central artery, patches of right ear pale; large veins pale. Does not flush as strongly as before; but strong difference between ears.

11.57   Left central artery relaxes slightly occasionally; but ear does not fill up like right.

1.30 p.m.   Left very pale, central artery just visible; slight fluctuation but never flushes; veins narrow; right flushes maximally after short period of blanching on excitation.

3.20   Left pale; right gorged maximally; striking difference.

4.00   Left central artery a mere thin line up to bifurcation at tip; beyond that moderately full; vein in upper inner part of outer surface fairly full, much fuller than before; left ear very pale; right ear usually gorged.

5.10   Left pale; central artery fairly visible; lateral veins not as prominent as before.   Right pales on excitation but swiftly fills up strongly, central artery fairly wide.

7.40   Left central artery now widens slighly; right ear flushes strongly and central artery dilates well; still good difference between ears.

10.35   Left ear now flushes maximally; after excitation both pale and then left begins to dilate promptly before right and reaches maximal engorgement before right. Right finally flushes just as much as left.

Summary.—In this experiment 0.4 cc. of adrenalin was injected on each side of the central artery and near it.   Shortly after the injection the entire artery began to constrict from below upward, the veins empty their contents a minute later. Eight hours after the injection there was still definite evidence of constriction.

But after twelve hours the injected ear showed a tendency to flushing. The non-injected ear seemed to have reacted with a tendency to flush after the injection of adrenalin into the other ear.

Experiment 9.—White female rabbit, 2570 grams. 10.42 a.m. Injected 1 cc. 0.9 per cent NaCl into base of the right ear; injected fluid spreads to central artery. Observed until 4.18 p.m. the same day and at 10.30 a.m. the next day.

Summary.—One cc. of NaCl 0.9 per cent injected into the base of the ear had no effect upon the width of the blood vessels of the ear, although the mass of the injected fluid reached the central artery.

Experiment 10.—(Full protocol.) Consists of observations made simultaneously on two rabbits. Rabbit A, white, 1500 grams and rabbit B, white, 1170. Each rabbit was given an injection of 1 cc. of a very dilute adrenalin solution into the base of the ear, 0.5 cc. on each side of the central artery and 5 mm. away from it. The solution was made up of 1 cc. adrenalin diluted to 100 cc. with 0.9 per cent NaCl equalling a solution of 1:100,000.

10.57 Injected 1 cc. of the dilute adrenalin solution into the base of the right ear of rabbit A.

10.58 Injected 1 cc. of the dilute adrenalin solution into the base of the left ear of rabbit B. Within one minute the injected ear was paler in each rabbit, but the blood vessels, central artery included, were by no means obliterated. Injected ear vessels were moderately dilated, but never reached the powerful engorgement seen in the normal ear. Excitation caused blanching of both ears.

11.05 Same; injected ears paler than normal ears, the difference is well marked but the central artery of the injected ear is moderately dilated (about one-half to one-fourth of size of the central artery of the normal ear).

11.22 Smaller vessels (arterioles) not as numerous in injected ear as in normal ears. Well marked difference between pale injected ears and flushed normal ears.

12.04 Well marked difference still present; vessels of injected ear never dilated to the same extent as in normal ears and smaller vessels never as numerous.

1.30 p.m. Rabbit A. Both ears blanch on excitation; then normal ear usually flushes first; later injected (right) ear begins to dilate until central artery is practically as large as that of normal ear, but injected ear nevertheless is paler, on account of the smaller arterioles not dilating as well as those of the normal ear.

Rabbit B. Difference between the two ears stronger than in rabbit A; injected ear does not fill up to the same degree as the normal ear, and central artery is always smaller.

3.05 Injected ears flush now about as strongly as control ears, often begin to flush before the normal ear after excitation. Central artery of injected ears as wide as those of control ears. In rabbit B upper fifth of central artery (including bifurcation) does not dilate well. This rabbit now responds but slightly to excitation: practically no blanching occurs.

5.40 Rabbit A. After excitation both ears pale at first then right (injected) ear flushed maximally before left, normal ear; after a considerable period normal ear dilated to same extent as injected ear. Well seen in central artery.

Rabbit B. Both pale at first examination, then both ears begin to gorge at approximately the same time; injected ear a bit more perhaps. Upper fifth of central artery now well dilated (was constricted before), while lower part of central artery contracts well.

Summary.—Experiment 10 consists of a double observation made simultaneously on two rabbits under exactly similar conditions. The constricting effect appeared swiftly in both animals, one minute after the injection. The constricting effect however, was at no time maximal, and the paleness depended largely upon the disappearance of the arterioles and less upon the constriction of the central artery, which was at no time excessive. The constricting effect lasted several hours. But in one animal the vessels of the injected ear showed an unmistakable tendency to dilatation four hours after the injection, while at that time in the other the condition of the vessels was on the borderline. In animal B the constricting effect seemed to be a trifle more persistent than in animal A.

Many more experiments of this sort were made. The results were essentially the same and are sufficiently illustrated by the experiments cited in the foregoing pages.

A number of experiments were made in which either the sympathetic nerve alone was cut or the superior cervical ganglion alone was excised, or in addition the large ear nerve was sectioned several days before the adrenalin experiment was made. The bloodvessels of the ears of rabbits so prepared were of course wider than in normal animals. But the course of the effect of an injection of adrenalin in these animals with denervated ear vessels was not materially different from that observed and described in the experiments on ear vessels with intact innervation.

We shall mention that in the various series of experiments we met with one only in which an injection of 0.3 cc. adrenalin at the inner margin of the base of the ear caused no perceptible effect upon the bloodvessels of that ear. The connective tissue in that place is dense and does not permit the spreading of the fluid to adjacent places. In all the other experiments the injection of adrenalin had a definite effect, the nature of which was invariably constriction of the bloodvessels.

<div align="center">DISCUSSION.</div>

Before discussing our results in detail, we wish to make a brief remark upon the restricted value of the bloodpressure curve as a means of studing the action of epinephrin. The rise of bloodpressure

following the intravenous injection of epinephrin gives information as to the constriction of the bloodvessels only by inference and not by direct observation, and does not tell the whole story. A rise in bloodpressure indicates that the vascular bed became smaller, which can be accomplished only by some constriction of bloodvessels. But the degree of constriction may vary in various parts of the body and so may its duration. Furthermore, an agent which causes a vasoconstriction in one part of the body may cause a vasodilatation in another part. The rise of bloodpressure is only an expression of the fact that the algebraic sum of the several effects of a given agent amounts to a diminution of the vascular bed of a certain degree and duration. The rise of bloodpressure, measured, as it is done, in the large arteries of the body, brought about by an intravenous injection of epinephrin, shows that the resultant is a vasoconstricting action. It does not indicate that there is another effect, and does not indicate what is the actual state of the bloodvessels of the heart, the kidneys, the bladder, the lungs, the brain, or of other parts of the body.

The intravenous injection of epinephrin which brings out the striking rise of bloodpressure, brings out at the same time another striking and puzzling fact, namely, the brevity of the rise. The duration of the rise varies with the injected dose and with the species of animals into which the injection is made (7). In rabbits in which the rise is the longest, the pressure returns to normal in seven minutes at the latest. As a rule, after return to normal the blood contains no demonstrable epinephrin.

At various times we met with experimental facts which showed that in some modes of adminstration the duration of the effect upon the bloodvessels is longer than the one observed after intravenous injections of epinephrin. In our second study of absorption from the intramuscular tissues (8) a tracing is reproduced which shows that about half an hour after the injection of adrenalin the blood pressure had not yet returned to normal. In our studies on the intraspinal injection of epinephrin (9) a tracing is published in which seventy-five minutes after an intraspinal injection the bloodpressure had not yet come down to the original level. In both instances the epinephrin was injected into places other than the vascular bed.

In the series of experiments reported in this paper the condition of the vessels was studied in a single organ, the ear; the injections were made into that organ itself subcutaneously, and the results were reached by a direct observation of the bloodvessels and the tissues under normal conditions. In these experiments we have seen nothing but constriction of the bloodvessels and even such a small dose of adrenalin as 0.01 mgm. caused an unmistakable constriction.

The outstanding fact in our results is the remarkable duration of the constricting effect. There was practically no instance in which the evident constriction of the vessels subsided in less than three hours and sometimes it persisted as long as eight hours. It must be emphasized that the maximum duration of bloodpressure rise by intravenous injection lasts seven minutes; the duration of constriction of bloodvessels by local subcutaneous injection of epinephrin lasts 180 to 480 minutes.

In general, it may be stated that the duration of the constricting effect of adrenalin increased with the size of the injected dose. When injected in the same place and under the same conditions the constricting effect lasted definitely longer after 1 cc., for instance, than after 0.5 cc. A very important factor is the place of injection, Compare for example the very long duration of constriction in experiment 8 (eight hours) in which 0.4 cc. was given near the central artery, on each side of it, with the duration in experiment 7 in which 1 cc. was injected midway between the central artery and the margin of the ear.

What was said for the duration holds true also, although perhaps to a less degree, for the intensity of the effect. The ear was paler and the vessels were more constricted, when the injected dose was larger and when the injection was made near the central artery.

The site of the injection had a special influence upon the latent period and the development of the constricting effect. When the injected fluid spread to the central artery, or, still more, when half of the quantity of solution was injected near each side of the central artery, paleness of the ear set in at once and in most instances with maximum intensity. In experiment 4, in which 0.5 cc. was injected midway between the central artery and the margin of the ear, more than ten minutes passed before there was any sign of constriction,

and during the entire experiment the effect was moderate. But in experiment 5, in which 0.25 cc. was injected on each side of the central artery and near it, the entire ear, from the base to the tip, became pale in less than one minute. The pallor was intense and the effect lasted about five hours. On the other hand in experiment 6, in which 0.3 cc. of adrenalin was injected on each side of the central artery but away from it and near the margin of the ear, the first constricting effect was noticed as late as twenty minutes after the injection, although gradually the effect became well pronounced and was of considerable duration. Or compare the following results. In experiment 7, 1 cc. of adrenalin was injected midway between the central artery and the margin of the ear. Eleven minutes passed before there was any sign of constriction; it then developed gradually but reached a considerable intensity and persisted with a gradually diminishing degree for about five hours. In experiment 8, 0.4 cc. was given on each side of the central artery. The pallor developed practically immediately, the constriction of the central artery was seen traveling up to the tip within one minute, and the effect was very strong and persisted for about eight hours.

Oliver and Schafer (10) assumed that medium sized arteries are not constricted by an adrenal extract and believed that they participate by passive dilatation in the oncometric rise of the curve obtained from an extremity. Langley (11) on the other hand thought that adrenal extract causes a constriction of the medium sized vessels. In our experiments there was not the slightest doubt as to the constriction of the central artery, which must be considered as middle sized. When a larger dose is injected and the injection is made at the base near the vessel, the entire artery becomes constricted at once and later may be invisible. (Sodium chloride, when injected at the base, exerts no effect.) With smaller doses and when injected at some distance from the artery, the constriction is gradual. In this case a chain of short fine sections of the artery, containing little columns of blood, may be seen repeatedly moving upward. The same is often seen when the constriction is on the point of receding. The relaxation of the central artery always sets in ahead of a noticeable subsidence of the pallor of the ear.

As far as we know, no study exists as to the action of epinephrin upon veins *in situ*. Gunn and Chavasse (12) stated recently that rings of veins contract when immersed in a solution of epinephrin. In our experiments we could have no doubt that the veins often become constricted; but the constriction of veins set in later than that of the central artery or of the arterioles. This was definitely seen even when the constriction of the central artery set in rapidly. When the development of the arterial constriction was a slow process, at first the blood in the veins became dark and the pale ear had a bluish hue; later the veins became empty; then they narrowed, began to lose their outlines and finally became invisible. In intense constrictions the effect extended also to the marginal veins.

The arterioles are seen as "ribs" coming from the central artery and a network of fine vessels is seen between the ribs in the more or less transparent areas on the sides of the central artery. When the injection was made near the artery, the arterioles disappeared practically simultaneously with the constriction of the central artery. When the injected fluid reached the artery only on one side the suddenness of the onset and the intensity of the effect upon the arterioles was greater on the side in which the injection was made. Apparently the central artery is bound to the surfaces by some dense tissue which offers resistance to the spread of the fluid. As the constricting effect progressed, not all the small arterioles disappeared at the same time, the "ribs" seemed to persist longer than the vascular network between them, and the ribs, too, disappeared only one after another. The decrease in the number of arterioles became evident before the constriction of the central artery made its definite appearance. (The first manifestation of an impairment of the central artery was its failure to dilate promptly after the constriction which follows an excitation or a struggle.)

The spaces in the ear between the arterioles are usually pink, which is due to the blood within the capillaries. When the constricting effect developed slowly the interarterial spaces seemed to lose their pink color before the arterioles began to disappear.

The effect of the injection of adrenalin became often manifest by some other signs than change or loss of color. *The ear became definitely cooler than the other ear, was heavier and hung down,*

while the other ear was erect. The ears were moved very little. With regard to the temperature, it was sometimes noticed that the injected ear felt cooler than the normal ear before it was recognized that it was paler.

We shall recapitulate some points. An injection of 0.3 cc. of adrenalin into the tip of the ear caused a rapid onset of a fairly intense paleness of the ear which was of long duration. An injection of 0.3 cc. of normal salt solution also caused a rapid onset of paleness, which however lasted only a short time. The early appearance of pallor in either case we ascribed, at least in part, to a mechanical factor resulting from the density of the connective tissue in the injected spaces. In the single failure mentioned above, the injection was made close to the margin of the ear where the connective tissue is quite tense, which prevents the fluid from spreading readily to adjacent spaces. The rapid effect from an injection in the tip was probably due mainly to the close proximity of the bifurcation of the central artery. The fluid ran down close to the artery, which was probably only *compressed* by the saline but became *constricted* by the adrenalin. It should be recalled that the injection of the saline caused the development of a ridge along the central artery, while no ridge appeared when adrenalin was injected.

When saline was injected at the base of the ear, even when the quantity was 1 cc., there was no effect upon the calibre of the vessels. When adrenalin was injected at the base there was an unmistakable constricting effect. When the injection was made on both sides of the central artery and near it, the paleness of the entire ear and the constriction of practically all the vessels developed rapidly and was fairly intense and of long duration. No ridge was ever observed to develop along the artery. When the injection was made midway between the artery and the margin of the ear, the effect developed more slowly and the slower the further away from the margin the injection was made. In this case some paleness developed in a good portion of the ear before the central artery showed any constriction; the latter progressed slowly along the central. artery.

Epinephrin is readily destroyed in an alkaline solution. With this in mind and with the fact of the alkalinity of the tissues, several authors ascribed the rapid decrease of the bloodpressure after an

intravenous injection of epinephrin to the escape of the latter into the body tissues and its destruction there. *Our experiments dispose completely of this explanation.* On the contrary, adrenalin injected directly into the tissues remained strongly active for many hours. It seems that our experiments rather favor the assumption that the place of destruction of epinephrin is within the bloodvessels. The constriction of the arterioles and capillaries prevents the rapid entrance of the epinephrin into the lumen of the vessels and thus permits its long continued efficient action. This presupposes that the constriction of the vessels in our experiments is caused by the epinephrin *reaching the tunica muscularis directly through the adventitia and not through the intima after entering the lumen of the vessels through the capillaries.* This deserves to be especially emphasized since some writers assume that all actions of epinephrin upon the bloodvessels are effected from the lumen of the vessels. Patta (13), for instance, claimed that the bloodpressure effect of subcutaneous or intramuscular injections is attained simply by the needle inadvertently entering a bloodvessel. In our previous experiments we (14) have taken special precaution to eliminate such accidents. In the present experiments the unusual length of the duration of the constricting effect eliminates the consideration of an intermediary action through the lumen of the vessels.

As to the rapid disappearance of the epinephrin within the blood, the experiments of Tatum (15) are interesting. He found that epinephrin disappears rapidly from a solution when arterial wall is present. But the solution has to be oxygenated. However, we shall not enter here into a discussion of the cause of the rapid disappearance of the epinephrin within the blood as well as the cause of the final disappearance of the constricting effect in our experiments.

The long duration of the constriction in our experiments is an interesting illustration of the low fatigability of the tunica muscularis. It is also a noteworthy fact that the paleness which persisted for many hours, and which sometimes was extreme, never led to necrosis or even to serious temporary injuries of the ear. While the place of injection was found sometimes next day to be slightly inflamed and edematous, it soon became normal again. Practically in all of our experiments the bloodvessels of the injected ear mani-

fested next day a tendency to dilatation which was definitely greater than in the vessels of the normal ear. Another point of interest is that in many experiments about simultaneously with the onset of the constricting effects observed in the injected ear, a greater dilatation of the vessels of the other ear made its appearance. This dilatation was not of very long duration. Both last mentioned phenomena may be significant; but we shall not attempt to analyze here their possible meaning.

SUMMARY.

Rise of bloodpressure is only a circumstantial evidence for the constriction of bloodvessels in some part of the vascular bed which is sufficient to overbalance any other effect. An ocular study of the bloodvessels in the rabbit's ears permits a direct observation of the behavior of all the larger vessels in that organ. The experiments reported in this paper have shown conclusively that a subcutaneous injection of epinephrin in the ear of rabbits causes a constriction of all the vessels of that ear. The constriction is quite intense; but the outstanding feature is its very considerable duration—between three to eight hours. The rise of bloodpressure from an intravascular injection of epinephrin is at the utmost seven minutes.

The latent period which passes between the time of the injection and the onset of the constriction is the longer the further away the injection is made from the central artery. Injections made near to the central artery and on both sides of it cause practically an immediate paling of the entire ear and constriction of the central artery with all its branches and of the veins.

In subcutaneous injections of the ear the epinephrin apparently reaches the muscular sheath through the adventitia and not through the intima from the lumen of the bloodvessels.

An ear which received a subcutaneous injection of epinephrin is cold, heavy, and is infrequently moved by the animal.

When the constriction passes off the bloodvessels which were subjected to this effect show later a tendency to the opposite effect, to vasodilatation.

A subcutaneous injection of adrenalin in one ear which causes a constriction of the vessels of that ear seems often to cause at about the same time a dilatation of the vessels in the other ear; the dilatation is not of long duration.

REFERENCES.

(1) OLIVER, GEORGE, AND SCHAFER, E. A.: Jour. Physiol., xviii, 277, 1895.

(2) MELTZER, S. J., AND MELTZER, CLARA: Amer. Jour. Physiol., ix, 57, 1903.

(3) MELTZER, S. J., AND MELTZER, CLARA: Amer. Jour. Physiol., ix, 147, 1903.

(4) MELTZER, S. J., AND MELTZER, CLARA: Amer. Jour. Physiol., ix, 252, 1903.

(5) MELTZER, S. J., AND MELTZER, CLARA: Jour. Med. Res., x, 185, 1903.

(6) MELTZER, S. J., AND AUER, JOHN: Jour. Exper. Med., vii, 1, 1905.
    AUER, JOHN, AND MELTZER, S. J.: Jour. Exper. Med., xiii. 328, 1911.

(7) GITHENS, T. S.: Jour. Exper. Med., xxv, 323, 1917.

(8) AUER, JOHN, AND MELTZER, S. J.: Jour. Exper. Med., xiii, 328, 1911.

(9) AUER, JOHN, AND MELTZER, S. J.: Amer. Jour. Physiol., xlviii, 286, 1918.

(10) OLIVER, GEORGE, AND SCHAFER, E. A.: Jour. Physiol., xviii, 277, 1895.

(11) LANGLEY, J. H.: Jour. Physiol., xxvii, 237, 1901-02.

(12) GUNN AND CHAVASSE: Proc. R. Soc., lxxxvi, 192, 1913.

(13) PATTA: Arch. ital. de biol., xlvi, 463, 1903.

(14) AUER, JOHN, AND MELTZER, S. J.: Jour. Exper. Med., xiii, 328, 1911.

(15) TATUM, A. L.: Jour. Pharm., iv, 151, 1912-13.

STUDIES ON DECREASING THE REACTION OF NORMAL SKIN TO DESTRUCTIVE DOSES OF X-RAYS BY PHARMACOLOGICAL MEANS AND ON THE MECHANISM INVOLVED.*

BY JOHN AUER, M.D., AND WILLIAM D. WITHERBEE, M.D.,

ROENTGENOLOGIST.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 100 TO 102.

In an earlier investigation evidence was presented to show that an organism sensitized by a foreign protein could locally autoinoculate itself with the same protein when certain conditions were fulfilled.[1] As this mechanism would serve to explain a number of abnormal reactions of hitherto cryptogenetic origin, it was desirable to advance still more proof. For this reason work was undertaken in which x-rays were the local irritating agent which caused the autoinoculation. During the preliminary stages of this research, however, an impression gradually developed that a certain group of rabbits seemed to show an increased resistance to doses of ordinarily destructive x-rays. Since this would be of considerable theoretical as well as practical value, if true, we abandoned our original object temporarily in order to study this point. The result demonstrated the correctness of the impression that the skin after the systemic incorporation of serum could be rendered resistant to doses of x-rays which are lethal to the tissues of non-prepared animals.

*A preliminary statement was recently published (Auer, J., and Witherbee, W. D., *J. Am. Med. Assn.*, 1921, lxxvi, 301).

[1] Auer, J., *J. Exp. Med.*, 1920, xxxii, 427.

Method.

An interruptorless, 10 kilowatt, 220 direct current machine with a medium focus Coolidge tube was used. After preliminary trials 30 skin units (Witherbee-Remer formula)[2] were chosen as the standard test dose of x-rays. This was produced by a 3 inch spark-gap, 10 milliampere current, 6 inch distance from target, and 20 minute exposure. All these factors were constantly controlled throughout the period of treatment of all the animals. No filter was employed, except that a disc of ordinary filing card was placed between the tube and the skin surface in order to reduce the heat effect.

Rabbits only were used. The area x-rayed was always 4 sq. cm. of the upper half of the right ear, the central artery of the ear passing through the middle of this space. The rest of the ear and body was protected by a sheathing of lead. Shifting of the x-rayed area, due to movements of the animal, was minimized by a simple device. The right ear was turned forward, smoothed out upon a small board and a strip of plaster fixed the tip of the ear to the board and the board to the box. A mask of sheet lead provided with an opening of 2 by 2 cm. was carefully placed in position on the right ear and held there by a strip of plaster. After covering the rest of the head and the entire box with lead sheeting, the animal was ready for treatment. Great care was exercised to prevent a circular constriction of the neck. This procedure was quite successful, though a moderate lateral shifting of the ear occurred in some instances.

It should be emphasized that the site chosen for x-raying offers a number of advantages: the ear is always easily available for inspection with no discomfort to the animal; two skin surfaces are affected by the x-rays, the dorsal on entry and the internal surface on exit of the x-rays; no serious systemic effects are to be feared even after massive doses because the x-raying is entirely localized to a comparatively small area; the ear of the rabbit is richly vascularized, and possesses a number of direct arteriovenous anastomoses[3] which guarantee an especially efficient collateral circulation.

All animals except the normal controls were x-rayed on the same day, but the members of no group were x-rayed in succession. The procedure was to take one animal from each group in rotation until all animals had been exposed to the x-rays.

After the rabbits had been x-rayed they were examined at 2 to 4 day intervals or daily when necessary, for a period of over 300 days, at which time the evidence was deemed sufficient to terminate this aspect of the work.

The end-point of the reaction was the appearance of a spot of dry gangrene in the x-rayed area, with subsequent fenestration. The number of days which elapsed between the time of x-raying and the appearance of dry gangrene, or the

---

[2] Witherbee, W. D., and Remer, J., *Arch. Dermatol. and Syphilol.*, 1920, i, 558; *Am. J. Roentgenol.*, 1920, vii, 485.

[3] Berlinerblau, F., *Arch. Anat. u. Physiol.*, 1875, 177.
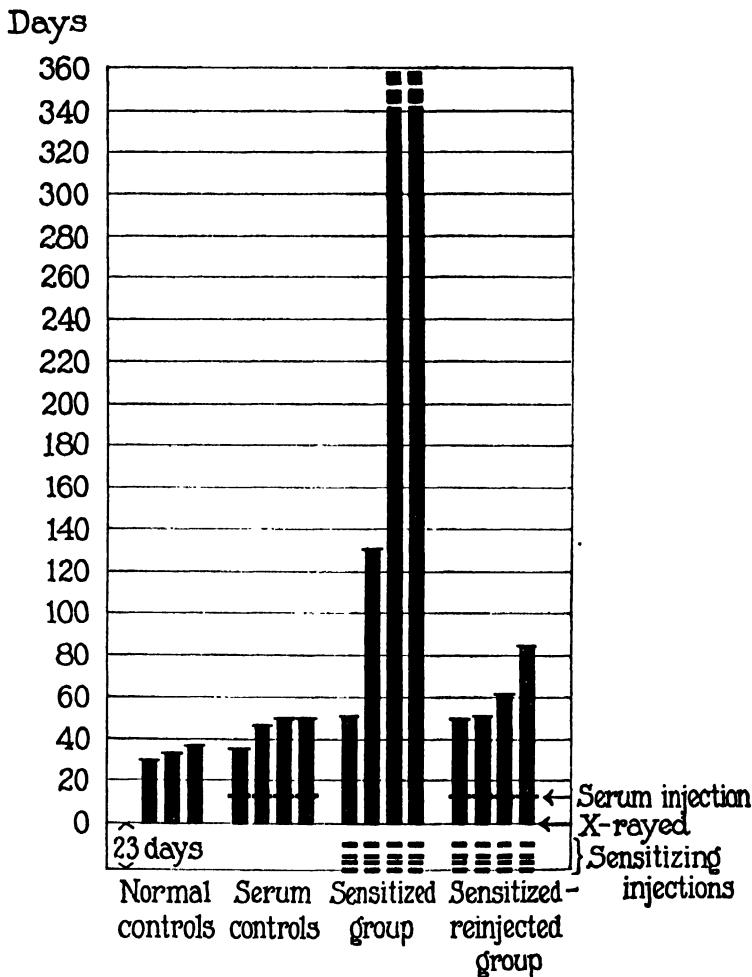
TEXT-FIG. 1. Duration of the reaction from the day of x-ray treatment to the appearance of perforating gangrene.

duration of the inflammatory process up to gangrene, was then plotted. Text-figs. 1 and 2 bring out well the striking difference between the various groups.

The type of rabbit employed, their feeding, care, method of injection, etc., have been described in an earlier paper.[1]
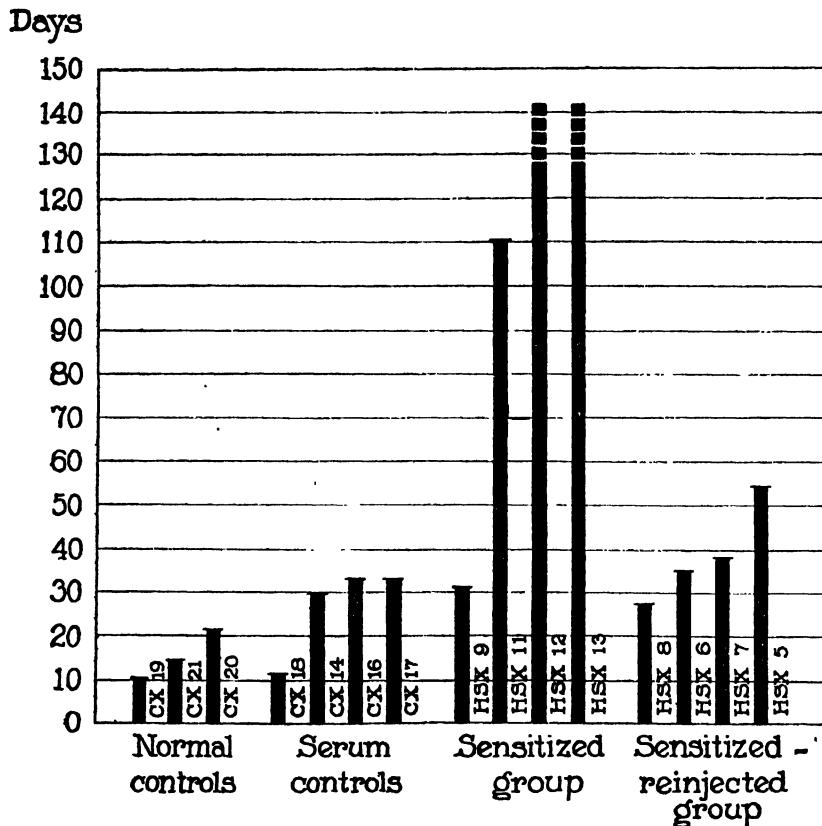
TEXT-FIG. 2. Duration of the reaction from the onset of the first exudate to the appearance of perforating gangrene.

The experimental animal material was composed of four groups of rabbits: (1) normal controls, (2) serum controls, (3) sensitized group, and (4) sensitized-reinjected group.

The normal controls were normal, untreated rabbits in which various doses of x-rays were tested as described. Both ears were utilized at different times. There were six rabbits in this group; in three the standard dose was used.

The serum controls, five in number, were normal rabbits which received a single injection of 10 cc. of horse serum[4] intraperitoneally, 13 days after the ear had been x-rayed.

The sensitized group of rabbits, five in number, was sensitized by two subcutaneous and two intramuscular injections of 1 cc. of horse serum each, at 3 to 4 day intervals. 10 days after the last sensitizing dose the right ears were x-rayed locally with the standard dose.

The sensitized-reinjected group, five rabbits, was prepared exactly as has been described for the sensitized group, but 13 days after x-ray treatment this group was reinjected intraperitoneally with 10 cc. of horse serum. This group was thus subjected to an anaphylactic reaction 23 days after the last sensitizing dose of serum. Text-fig. 1 shows the relation of the groups and the various procedures.

During the early stages of the work one rabbit in each of the last three groups died without obvious lesions. These groups therefore now consisted of four rabbits each.

RESULTS.

Before presenting the results in detail we shall first give the main outstanding facts of this work.

The normal control rabbits developed dry gangrene and fenestration in the x-rayed area in 30, 33, and 37 days respectively.

The serum control rabbits showed dry gangrene and fenestration in the x-rayed ears after 36, 47, 50, and 50 days respectively (Figs. 1 and 2).

The sensitized-reinjected group exhibited the same lesions after 50, 52, 62, and 85 days respectively (Figs. 5 and 6).

The sensitized group, however, responded quite differently on the whole. Only one rabbit showed gangrene and fenestration of the ear in 46 days. A second rabbit developed the same lesion, but only after 131 days. The two remaining rabbits have developed no gangrene or fenestration even after the lapse of more than 340 days (Text-figs. 1 and 2 and Figs. 3 and 4).

These data demonstrate clearly that rabbits previously sensitized by the parenteral injection of horse serum acquire a remarkably increased resistance in the majority of instances to doses of x-rays which are lethal to the tissues of normal control rabbits or serum control rabbits.

[4] The horse serum was kindly furnished by Dr. W. H. Park and Dr. E. J. Banzhaf of the Department of Health of the City of New York.

The results also show convincingly that the protection which serum sensitization previous to x-ray treatment confers is largely abolished when the sensitized and x-rayed animals are subjected to a general anaphylactic reaction (Text-figs. 1 and 2, sensitized-reinjected group).

The main objective details of the investigation are as follows: Within 24 hours after x-ray treatment two to three rabbits out of each group of five showed a slight pinkness of the x-rayed area which disappeared within 2 to 3 days. This pinkness is probably due to a heat effect from the Coolidge tube. Within 4 days the hair of the x-rayed area began to loosen, though there was considerable variation. Thus for example on the 11th day after x-ray treatment some ears showed bald patches, while in others the hair was still firmly fixed. This variation bore no relation apparently to the experimental group. Pigmentation of the x-rayed area was noticeable in 2 to 4 days after x-ray treatment and varied with the different animals. In some it became very marked, while in others the pigmentation was always slight. The degree of pigmentation bore no definite relation to the group to which the animal belonged. A slight thickening of the x-rayed area, without any obvious vascular congestion, was first noticed 9 days after x-ray treatment; it occurred in one to three rabbits of each group. After 11 days these rabbits showed, in addition to the thickening, a slight but definite congestion of the x-rayed area. 13 days after x-ray treatment all rabbits, except two members of the sensitized-reinjected group, showed a definite though slight congestion with slight thickening of the x-rayed area. It should be noted that no rabbit had yet been reinjected with serum at the time of this examination.

The congestion of the x-rayed area increased slowly, but not at an equal rate in all the groups. Thus, 16 days after x-ray treatment the serum control rabbits still exhibited only a slight congestion of the x-rayed area, while the sensitized group showed a moderate to marked congestion, and the sensitized-reinjected animals a fair to moderate congestion. Associated with the increased congestion there was also a slight increase in the thickness of the x-rayed patch.

After 18 to 25 days generally the x-rayed area developed an exudate on both surfaces which dried into crusts. The healing of this first inflammation was usually complete within 28 to 36 days and the x-rayed areas now appeared like healed superficial wounds. The x-rayed area was absolutely bald and practically free of crusts; the skin was thin, whitish, glistening, and easily crinkled into thin folds; the blood supply was good, though many rabbits showed pearly white spots in the x-rayed areas; there was no gangrene.

This termination of what we shall call the first inflammation did not take place in all rabbits, but occurred in a majority of the serum controls, the sensitized group, and the sensitized-reinjected group (Table I). In the normal controls (three rabbits) this first inflammation with crusts did not clear up but passed at once to a complete perforating gangrene. The same fact was also observed once in the serum control group (No. CX 18) and once in the sensitized-reinjected group (No. HSX 8) (Table I).

TABLE I.

Group.	Series No.	First inflammation.		Interval between first and second inflammation.	Second inflammation.		Delayed second inflammation.	Length of observation.
		Day of onset of exudate.	Day of healing.		Day of onset of exudate.	Day of appearance of gangrene.		
				days				*days*
Normal controls.	CX 19	22nd	No healing before gangrene.			33rd	—	69
	CX 20	16th	" " "			37th	—	69
	CX 21	16th	" " "			30th	—	69
Serum controls.	CX 14	18th	28th	8	36th	46th–49th	0	300+
	CX 16	18th	36th	6	42nd	49th–52nd	0	300+
	CX 17	18th	36th	13	49th	49th–52nd	0	300+
	CX 18	25th	No healing before gangrene.			36th	171st–195th	300+
Sensitized group.	HSX 9	15th	28th	14	42nd	46th	0	300+
	HSX 11	20th	28th	68	96th	128th–135th	0	300+
	HSX 12	20th	36th	At least 340.	0	0	0	300+
	HSX 13	23rd	28th	At least 340.	0	0	0	300+
Sensitized and reinjected group.	HSX 5	31st	42nd	10	52nd	82nd–89th	0	300+
	HSX 6	16th	36th	13	49th	49th–52nd	0	300+
	HSX 7	25th	49th	3	52nd	61st–64th	0	300+
	HSX 8	25th	No healing before gangrene.			52nd	0	300+

The figures represent the number of days after x-ray treatment unless otherwise stated. In all rabbits 4 sq. cm. of the right ear were x-rayed with 30 skin units.

The recovery from the first inflammation and the disappearance of the crusts were, however, not permanent in all the rabbits. After a period during which the x-rayed areas looked like healed or practically healed surface wounds, another exudate appeared unexpectedly on these x-rayed surfaces. This typical, second inflammation always led to a perforating gangrene (Table I). The interval elapsing between the end of the first inflammation and the onset of the second inflammation varied from 3 to 13 days in the serum controls and the sensitized-reinjected group. In the sensitized group, on the other hand, the interval before the second inflammation appeared was 14 days in one rabbit, 68 days in a second, and in the two remaining rabbits no second inflammation leading to gangrene has developed after more than 340 days (Table I and Text-fig. 1).

The character of the second inflammation occurring in the x-rayed area was interesting. A fairly marked redness and swelling of the x-rayed tissue preceding gangrene were observed only once (No. HSX 11), and in all the other rabbits in which fenestration of the ear developed, the inflammatory signs were a moderate pinkness of the x-rayed tissue with no definite swelling or heat; the most noticeable feature was the appearance of a sticky exudate, often on both ear surfaces. This exudate apparently was poured out at various rates, for the subsequent crusts exhibited a definite lamination[5] in many instances.

The difference between the inflammatory reaction of the x-rayed tissue and the normal surrounding tissue was well illustrated in four animals, one in each group—Nos. CX 21, CX 14, HSX 11, and HSX 8 (Table I). In these rabbits an inflammation of the right ear set in, perhaps due to scratching. This inflammation was most marked around the periphery of the x-rayed area, especially at the upper and lower borders. In the untreated part the tissues were red, swollen, hot, and in two rabbits (Nos. HSX 11 and HSX 8) a tongue of edema ran from the lower border of the x-rayed area towards the base of the ear; the blood vessels of the untreated part of the ear were markedly dilated. The inflamed tissue, however, stopped sharply at the x-rayed area, and this latter tissue, while more or less pink in all, stood out in striking contrast to the inflamed surroundings, which thus framed the comparatively pallid x-rayed area. The central artery, turgid with blood above and below the x-rayed area, was a mere red thread within this space. There was no appreciable thickening of the x-rayed area on palpation except in No. HSX 11, in which a well marked edema of the lower portion set in. The second inflammation of the x-rayed area was therefore in general of a definite subacute character, while the first inflammation resembled a mild, acute inflammation.

Another striking difference between the x-rayed and normal tissues was observed in the development of exudate and crusts in the four rabbits mentioned above. The marked inflammation of the normal ear tissue did not lead to exudate and crust formation, while thick crusts often developed in the x-rayed areas.

---

[5] This lamination was observed in the secondary exudate; no notes were made on the structure of the crusts in the primary exudate.

The chief sign which heralded the onset of a perforating gangrene was the appearance of a small, brown-black, slightly sunken spot on the internal skin surface of the x-rayed area; occasionally an exceptionally thick crust was the first sign. In the last three groups the sunken, discolored spot was noted seven times in the ten rabbits in which fenestration took place. The appearance of this discoloration, however, did not invariably foretell the onset of a perforating gangrene. Thus No. HSX 13 exhibited a brown discoloration of the internal surface with slight, thin crust formation 64 days after x-ray treatment. This lesion did not progress, but was practically healed on the 86th day. It will be remembered that this rabbit belongs to the sensitized group and showed no gangrene of the x-rayed area within more than 340 days after x-ray treatment (Table I and Text-fig. 1).

The initial point where a perforating gangrene developed was small when first observed, at times not more than 1 to 2 mm. in diameter. This dry spot then increased in size, first rapidly, then slowly until an equilibrium was established between the destructive and reparative factors. Several times two spots of gangrene developed, one on each side of and close to the central artery of the ear. These two spots always fused sooner or later, but the gangrenous process was always more rapid away from the artery than towards it, though finally the intervening section of the artery also dried up.

The amount of tissue lost by gangrene was never equal to the entire area x-rayed; in only two instances did the loss exceed 50 per cent. The gangrenous process began near the center of the area x-rayed and then progressed towards the borders. This spread was usually greater in the lateral direction than towards the root or apex of the ear. In the serum control group the loss of tissue varied between 80 and 130 sq. mm.;[6] in the sensitized group (two rabbits) between 117 and 224 sq. mm.; and in the sensitized-reinjected group the loss fluctuated between 70 and 210 sq. mm.

The measurements given are only rough approximations of the losses, and no effort was made to determine the true areas of the more or less irregular fenestrations. We believe, however, that the figures convey a just impression.

In addition to the second, subacute type of inflammation leading to gangrene, which has been described, a delayed, second form of subacute inflammation also leading to gangrene may be distinguished. This form was observed only once; it occurred in Rabbit CX 18 of the serum control group (Table I). In this rabbit the first inflammation beginning 25 days after x-ray treatment led at once to a perforating gangrene 36 days after treatment. A similar acceleration of the process took place in No. HSX 8 of the sensitized-reinjected group and in all the

---

[6] No measurements were made in the last rabbit, No. CX 18, because the gangrene involved the border of the ear, due to a shift during x-ray treatment. Here also the loss was less than 50 per cent of the x-rayed area. The loss of tissue sustained by the normal controls was not measured, due to an oversight.

normal controls (Table I). In No. CX 18, however, a subacute inflammation leading again to gangrene developed 135 days after the first. During this interval of time the remainder of the x-rayed area did not exhibit any obvious differences from the x-rayed areas of other rabbits.

Final Changes in the X-Rayed Area.

When the x-rayed areas of all the rabbits are examined some months after the last inflammation, all of them, including Rabbits HSX 12 and HSX 13 which never developed a perforating gangrene, show a number of changes in common. In all, the x-rayed area is hairless, the skin covering this area is smooth with perhaps a slight branny desquamation on the dorsal surface, and this skin wrinkles readily into thin folds. Occasionally, especially on the internal surface, numerous small, oval, yellowish brown thickenings of the outer skin are observable. These are less than 1 mm. in diameter and still less in thickness. They are seated in cup-shaped depressions of the skin, and probably represent keratoses.

The borders of the fenestrations generally show little or no thickening, but at or near the fenestration one or more red or reddish brown, slightly elevated papules are observable. These papules are formed by a number of dilated, small blood vessels. Occasionally, a slight hemorrhage proceeds from the angiectasias and the blood may burrow under the outer layers of the skin epithelium. These small masses of dilated blood vessels were also observed in Rabbit HSX 13 in which no perforating gangrene occurred; they were not seen in Rabbit HSX 12 of the same group.

In addition to these angiectasias the x-rayed areas show a number of tortuous blood vessels; often they are especially evident upon the internal surface. In the two rabbits of the sensitized group, Nos. HSX 12 and HSX 13, in which gangrene of the x-rayed area did not develop, these tortuous blood vessels are especially noticeable about the neighborhood of the central artery, where they form a delicate tracery of blood channels which are apparently superficial. The central artery itself in the x-rayed area of these two rabbits is narrow, slightly irregular in outline, and looks blurred in that portion of its course where the angiectasias are most marked.

The blood vessels of the healed, x-rayed areas do not react normally. In the normal rabbit the ear vessels respond by an initial blanching when the animal is sharply tapped, or a moderate struggle is induced; this blanching is followed by a marked vasodilatation if the original stimulus was sufficiently strong and if the room is not too cold. In the x-rayed areas of the experimental rabbits, however, this test causes at first some increase in the pallor, which later is replaced by only a slight dilatation of both arteries and veins. This striking difference in the vasomotor response of the x-rayed and untreated ears is well illustrated by Figs. 1 to 6, which were obtained by photographing the ears of two members of each group of rabbits during the stage of vasodilatation. It will be noticed that

both arteries and veins show a definite narrowing of caliber on entry of the x-rayed area, and that the original caliber is largely if not entirely regained when these vessels issue from the x-rayed area.

DISCUSSION.

From the experimental facts described above and summarized in the table and charts, it is clearly evident that the skin of rabbits under certain conditions may acquire a remarkably increased resistance to doses of x-rays which are surely destructive to control animals. These conditions are that the animal whose skin tolerance to x-rays is to be increased must be sensitized with horse serum and this sensitization must take place before the rabbit is exposed to the x-rays.

The evidence for these conclusions is summarized in Text-figs. 1 and 2. In these charts it is shown that the standard dose of 30 skin units of x-rays causes a perforating gangrene of the ear in normal controls within 37 days after x-ray treatment. The same dose of x-rays administered to the ear of rabbits previously sensitized with horse serum (sensitized group in the chart) was, however, remarkably weakened in its effect upon the tissues exposed to the x-rays. Two animals showed no gangrene at all during the period of examination (over 340 days); one exhibited a perforating gangrene after the lapse of 131 days, and only one member of the group of four rabbits reacted fairly like the normal controls by developing a perforating gangrene in 46 days. That sensitization must be present before the animal is exposed to the standard test dose of x-rays, if protection from the ordinarily destructive effects of this dose is desired, is shown by the serum control group. These rabbits were normal animals and were injected with horse serum for the first time, but this injection took place 13 days after exposure to the x-rays. In this group all rabbits developed fenestration of the ears subsequent to dry gangrene within 50 days after exposure to the x-rays. The serum injection after x-ray treatment therefore conferred no marked trace of protection to the x-rayed areas of the ears.

Additional evidence to establish this point, that sensitization previous to x-ray treatment confers a marked increase in resistance, is furnished by the behavior of the x-rayed area in Rabbit HSX 13 of the sensitized group. In this animal the x-rayed area a number of times exhibited some crust formation with moderate congestion of the surrounding vessels. In addition, the internal surface presented a brownish, sunken discoloration such as frequently preceded the appearance of a perforating gangrene in the x-rayed areas of the control rabbits. Yet healing was fairly prompt and no perforating gangrene resulted. The recuperative power of this x-rayed area, therefore, was greater than that existing in the x-rayed areas of the controls.

Another observation which points to the same conclusion is the inflammatory reaction which occurred previous to fenestration within a portion of the x-rayed area of a sensitized rabbit, No. IISX 11.   This inflammatory reaction was accompanied by a fair degree of redness and swelling and was much more pronounced than that observed in the x-rayed area of any other rabbit, though it was considerably less than the inflammation which the same rabbit showed in the adjoining untreated portion of the ear.   This increased inflammatory response can only be interpreted as an expression of a more vigorous state of this x-rayed area when compared to that of rabbits of other groups.

Another fact which seems clear is that the protection to x-rays which sensitization previous to x-ray treatment gives, is largely abolished if these animals are reinjected with serum after being x-rayed; in other words, if they are subjected to an anaphylactic reaction.

The evidence for this statement is summarized in Text-figs. 1 and 2.   The sensitized and reinjected group, it will be seen, was treated exactly like the sensitized group with but one exception: 13 days after being x-rayed and 23 days after the last sensitizing dose of serum, this group was reinjected with horse serum, and in consequence a mild, general anaphylactic reaction resulted, from which all promptly recovered.   Nevertheless, the further course of the experiment showed that these reinjected rabbits had largely lost the protection which mere sensitization gives (see the sensitized group, Text-figs. 1 and 2), and dry gangrene with fenestration took place in due time.   That some protection had still remained, however, is indicated by the fact that the interval between x-ray treatment and gangrene is appreciably longer in two animals (62 and 85 days respectively) than in any of the controls (see also Text-fig. 2).

The increased resistance of skin-covered tissues to unfiltered x-rays which results from previous sensitization with an undenatured protein may be roughly estimated from our data.   In preliminary experiments we tested the effects of 15, 18, and $22\frac{2}{3}$ skin units of x-rays on areas of rabbits' ears 4 sq. cm. in size.   With $22\frac{2}{3}$ units, perforating gangrene occurred in the two rabbits tested within 37 to 43 days.   With 18 skin units, perforating gangrene took place 58 days after x-ray treatment in one rabbit, and incomplete gangrene in two others after 72 days, when observations were discontinued. With 15 skin units complete gangrene occurred in one rabbit after 91 days, incomplete gangrene in a second after 91 days, and no gangrene at all in a third animal after the same interval, when, unfortunately, all these rabbits were discarded.

From these incomplete data we may nevertheless conclude that sensitization with horse serum previous to x-ray treatment can reduce, at least in some animals, the destructive value of 30 skin units of x-rays to a level of 15 to 18 skin units.

It must not be forgotten that the conclusions which we have drawn so far rest upon experimental evidence gained under specific conditions which have been described in detail above. Further work must show whether modifications of these conditions entail significant changes in the result.

Our knowledge concerning the various factors involved is limited. We do not know fully what influence the degree of sensitization plays; whether or not a phase of increased susceptibility to the action of x-rays precedes the establishment of a heightened resistance; how long this increased resistance persists; what the maximum resistance is which can be attained by this procedure, and other questions.

To some of these questions a partial answer can be given. As far as the degree of sensitization is concerned, one may state that the rabbits employed were highly sensitized. In earlier series of experiments, the same sensitizing procedure, dose, and period of incubation had been used by one of us, and in there animals the intravenous reinjection test had yielded a high mortality rate. It must always be remembered, however, that the degree of sensitization which a certain fixed method achieves, fluctuates more widely in rabbits than in guinea pigs. This may explain why we failed to produce any sign of protection in one rabbit of the sensitized group (Text-figs. 1 and 2).

As far as the maximum amount of x-rays is concerned, our results with 30 skin units indicate that this dose is fairly close to the limit of tolerance with the experimental procedure employed.

Specificity.

The increased resistance to ordinarily lethal doses of x-rays which tissues may gain after a preliminary treatment with an undenatured foreign protein must be classed as a non-specific reaction, because the altered, abnormal response is called forth not by the sensitizing substance but by an utterly unrelated, physical agent. Such non-specific reactions after sensitization have been described and recognized for years. Heilner[7] in 1908 observed that serum-sensitized rabbits succumbed to an injection of 4 per cent sodium chloride which was practically harmless to normal controls. Davidsohn and

[7] Heilner, E., *Z. Biol.*, 1908, 1, 487.

Friedemann[8] showed that rabbits sensitized with bovine serum react with temperature elevations to doses of sodium chloride given subcutaneously or intravenously, which produce no such effect in normal rabbits. Richet[9] noted that dogs sensitized by actino-congestine or crépito-congestine vomited after smaller doses of apomorphine hydrochloride, injected intraperitoneally, than normal dogs.

Non-specific reactions have also been utilized therapeutically,[10] but in this respect great caution is advisable. It must be realized that the incorporation of an undenatured foreign protein entails consequences of whose manifestations we are largely ignorant, and therefore often no intelligent balance can be struck between the harm and benefit which the procedure affords the patient. This deficiency in our knowledge should theoretically be lessened by laboratory work on the lower animals. For these reasons the irrational use of vaccines and sera is to be discouraged. Such powerful drugs should be used only when nothing else suffices to gain the desired therapeutic end. A conscious distinction should be drawn between drugs whose single injection exerts a comparatively short effect and those whose single injection releases reactions which are often masked and persist for months and even years. Sera and vaccines therefore may not be employed with the same careless freedom, which, for example, characterizes the use of various synthetic compounds.

Mechanism of Protection.

From the experimental data already presented and from the more obvious conclusions which we have so far drawn, no understanding of the underlying mechanism which brings about this increased resistance of the tissues to x-ray destruction can be reached. Some such

[8] Davidsohn, H., and Friedemann, U., *Berl. klin. Woch.*, 1909, xlvi, 1120; *Arch. Hyg.*, 1909, lxxi, 42.

[9] Richet, C., *Compt. rend. Soc. biol.*, 1910, lxviii, 820.

[10] For a review see Jobling, J. W., *The Harvey Lectures*, 1916–17, xii, 181; Miller, J. L., *J. Am. Med. Assn.*, 1921, lxxvi, 308; Cowie, D. M., *J. Am. Med. Assn.*, 1921, lxxvi, 310; Culver, H., *J. Am. Med. Assn.*, 1921, lxxvi, 311; Petersen, W. F., *J. Am. Med. Assn.*, 1921, lxxvi, 312.

basis can be obtained, however, if the results are considered in the light of a broad generalization of anaphylaxis. Such a generalization is the experimentally founded view[11] that an anaphylactic reaction is initiated when the anaphylactic antibody comes into contact with its antigen, during which process both antigen and antibody largely if not entirely disappear. If Text-figs. 1 and 2 are examined it will be observed that the only difference existing between the sensitized group and the sensitized-reinjected group is that the latter was subjected to an anaphylactic reaction 13 days after the x-ray treatment. In the sensitized-reinjected group, therefore, the anaphylactic antibodies had been removed more or less, while they were still present abundantly in the sensitized group. Since a majority of the sensitized group showed the marked resistance to massive doses of x-rays, while in the sensitized-reinjected group gangrene took place in the x-rayed area, it may be inferred that this protection is attributable to the anaphylactic antibodies which are present in the rabbits of the sensitized group, but which are not present, or at least not to the same functional degree, in the sensitized-reinjected group.

If the anaphylactic antibody is responsible for the protection to x-rays which the ears of the sensitized group exhibited, another inference may be drawn due to the fact that the x-ray treatment in the experiments was local. It follows that this protection must be assigned to the antibodies which are anchored to the tissue cells exposed to the x-rays and not to the circulating antibodies. This is shown clearly by the animals in the serum control group (Text-figs. 1 and 2). These animals had received one injection of horse serum, but this had been administered 13 days after the local x-raying of the ear. Within a short period an abundance of specific antibodies must have appeared in the circulation, and these necessarily must have traversed the capillary system of the x-rayed area of the ear. Yet practically no protection was conferred.[12] It appears, therefore, that the cells

[11] Anderson, J. F., and Frost, W. H., *J. Med. Research*, 1910, xxiii, 44; see also review by Wells, H. G., *Physiol. Rev.*, 1921, i, 51.

[12] A slight degree of protection is probably present. As shown in Text-fig. 1, the serum controls developed fenestration of the ears in 36 to 50 days while the normal controls attained the same state in a shorter time, 30 to 37 days. See other evidence in Text-fig. 2.

of an x-rayed area are unable to produce anaphylactic antibodies or to fix them, when present in the circulation, in sufficient amount to protect, provided that the x-ray treatment takes place before the injection of the antigen. The sensitized-reinjected group also supports this inference; in this group the antibodies formed as a result of the second injection of serum did not adequately replace those which were originally fixed in the x-rayed area but which were rendered inert by the anaphylactic reaction, though a certain measure of protection was observed (Text-figs. 1 and 2).

Finally, it may be inferred that the locally fixed anaphylactic antibodies (sensitized group) can be functionally removed by an anaphylactic reaction (sensitized-reinjected group) and the local protection which these fixed bodies gave against massive doses of x-rays is then largely abolished.[13]

It is probable that the increased resistance to x-rays conferred by a previous sensitization to the skin of rabbits will also be obtainable in man, and the procedure may thus be of utility in human therapeutics. Such a contingency will appear when malignant growths must be treated without the scalpel. Under these conditions the applicable dose of x-rays is directly limited by the resistance of the skin overlying the neoplasm for example, and a lethal dose for the cancerous tissue perhaps cannot be applied because it would also destroy the integument. This tentative proposal presupposes that the cancerous tissue does not acquire the same degree of resistance as the skin cells after sensitization, also that the heavy doses of x-rays do not ultimately produce malignant skin alterations. Concerning the first supposition, there is no knowledge available at present, but the experimental test is easily made; concerning the second, it may be said that no malignant changes in the skin of rabbits have been observed after a period of more than 300 days.[14] Finally, it may be stated that no objection can be urged against the parenteral employment of an undenatured foreign protein in such cases, because this effort is perhaps a last scientific attempt to help and it is therefore legitimate for the physician to invoke the aid of the protein molecule, fully conscious though he is that some or many of its various effects are not wholly desirable.

---

[13] It is impossible to decide whether the moderate resistance of the sensitized-reinjected group is due to an imperfect removal of the anchored antibodies during the anaphylactic reaction or to anchorage of some antibodies subsequent to the anaphylactic reaction.

[14] This period of time in the rabbit is comparable to a much longer interval in the human subject, if we consider the relative length of life in the two species.

That sensitization with a foreign protein protects the skin from the harmful effects of a subsequent x-ray treatment is indicated by studies made by Hektoen. In a series of important observations[15] Hektoen studied the effect of massive doses of x-rays under various conditions upon the production of antibodies, the anaphylactic antibody not being included. His experimental material consisted of white rats, rabbits, and dogs, and the entire body of the animal was always subjected to the action of the x-rays. Hektoen established clearly that the time of x-raying with respect to the injection of antigen exerted a profound effect upon the antibodies. If the antigen was injected immediately after the preparatory x-ray treatment the production of antibodies was practically completely restrained. If, on the other hand, the x-raying was carried out at the height of antibody production (days or weeks after the antigen injection) no effect was noted on the antibody output.

The observations which interested us most, however, were as follows: When young puppies were x-rayed with strong doses of x-rays before they were injected with antigen (10 per cent rat or goat blood suspensions), severe burns of the skin were noted;[16] but if they were x-rayed about 7 days after the antigen injection, Hektoen apparently observed no burns, for he only states that now many dogs showed no disturbances of the general health.[17] If we are correct in this interpretation of Hektoen's work, our observations in this matter accord with his. We have not been able to find any other observations in the literature bearing upon this question.

Inflammation of the X-Rayed Area.

In the objective record of our results we have described three combinations in which inflammation of the x-rayed ear surface may appear. These three combinations, their distribution among the various experimental groups, and the duration of the process can be utilized to give further support to the antibody theory which has already been discussed.[18]

[15] Hektoen, L., *J. Infect. Dis.*, 1918, xxii, 28. This article gives the references to Hektoen's earlier work. See also Hektoen, L., *J. Infect. Dis.*, 1920, xxvii, 23.

[16] Hektoen, L., *J. Infect. Dis.*, 1918, xxii, 29.

[17] Hektoen,[16] p. 31.

[18] We have not included among the various types of inflammatory reaction the combination noted in Rabbit CX 18 (Table I). In this serum control rabbit the first inflammation exceptionally led at once to complete gangrene of a section of the x-rayed area. But 135 days later a subacute inflammation with crust formation developed and led to still another loss of tissue. This delayed second inflammation healed in 24 days (195 days after x-ray treatment). This type of reaction is probably allied to the delayed x-ray lesions which at times occur in the human subject months after the last treatment (Pfoerringer, S., Review in *Am. J. Roentgenol.*, 1917, iv, 642).

The three combinations of states are as follows:[19]

(1) First inflammation................................gangrene.
(2) First inflammation...healing......second inflammation....gangrene.
(3) First inflammation................................healing.

The distribution of these combinations is summarized in Table I. An examination of this table shows that the second type or combination (first inflammation—healing—second inflammation—gangrene) occurs only in the groups which had been subjected at one time or another to the injection of horse serum. It was never observed in normal rabbits treated with a destructive dose of x-rays.

The first combination (first inflammation—gangrene) occurred in all of the three normal controls; it also was observed in two additional normal control animals which had been x-rayed with 22⅘ skin units. But in the serum animals this combination was only noted two times (Rabbits CX 18 and HSX 8).

The third combination (first inflammation—healing) was only observed in the sensitized group, in which the horse serum was administered previous to x-ray treatment. It occurred two times out of four experiments, in Rabbits HSX 12 and HSX 13. A third rabbit of this group (No. HSX 11) shows a very marked prolongation in the interval between recovery from the first inflammation and the onset of the second inflammation which led to gangrene.

From the occurrence of the second type of combination (inflammation—healing—inflammation—gangrene) in eight out of twelve rabbits which had been treated with horse serum (Table I), and from the failure of this combination to appear in five normal control animals to which no serum had been given, we may infer that the increased resistance of the x-rayed tissue evinced by the second combination of states is definitely ascribable to the serum treatment. In other words, the administration of serum at any time within the limits employed in the experiments changes the reaction of the x-rayed tissue from the first combination (inflammation—gangrene) to the second combination (first inflammation—healing—second inflammation—gangrene) in the majority of the treated rabbits.

[19] It should be remembered that the first inflammation was a mild acute form, while the second inflammation was subacute in character.

We conclude, therefore, that this change was due to a protective antibody action which was produced by the parenterally injected horse serum. From the data given in this section no inference can be drawn concerning the type of antibody which caused this change of reaction to the standard dose of x-rays. Such an inference, however, can readily be drawn if we use the occurrence of gangrene and the duration of the entire process (Text-figs. 1 and 2) as criteria, and in a preceding section evidence has been presented that the anchored, anaphylactic antibody may be considered the protective factor. It is therefore probable that the same anchored anaphylactic antibody is also responsible for the altered character of the local reaction which the serum-treated rabbits exhibit after x-ray treatment. What part, if any, is played by other types of antibodies in this matter cannot be determined by the data at hand.

On the basis of these considerations the various successions of conditions observed in the x-rayed areas of the rabbits may be explained as follows: The inflammation observed in normal control animals which ends in gangrene is the normal slow, destructive action of our standard x-ray dose (30 skin units). The tissues exposed show a mild, acute inflammation which leads to a complete destruction of a portion of the x-rayed area. How these tissue changes are produced by the physical agent, the x-rays, we do not know;[20] vascular changes such as we have described undoubtedly are involved in the process.

If rabbits are treated with horse serum parenterally and exposed to the same standard x-ray dose, the type of reaction changes, due to the presence of anaphylactic reaction bodies anchored in the x-rayed area, the latter factor depending upon the time or times when the serum is administered. If the serum is administered about 2 weeks after x-ray treatment or if it is injected before and after x-ray treatment in such a way that a general anaphylactic reaction results, the second combination of conditions (inflammation—healing—inflammation—gangrene) then appears in the x-rayed areas of a majority of the rabbits (Table I, serum control group, sensitized-reinjected group).

[20] For a good presentation of the various theories concerning the mode of x-ray action upon tissues, see Hall, C. C., and Whipple, G. H., *Am. J. Med. Sc.,* 1919, clvii, 455.

The first inflammation now progresses to healing due to the presence of anaphylactic antibodies anchored in the x-rayed area. But this healing is only temporary, because the amount of locally available antibodies is too small or becomes functionally inert, and the slowly acting destructive forces gain the ascendancy over the reparative agencies. As a consequence the second inflammation appears which leads to a perforating gangrene. The second inflammation is subacute in character because the exposed area has been damaged by the x-rays, so that it can no longer react acutely to an inflammatory stimulus.

If, however, the serum is administered to a rabbit about 10 days previous to exposure to the standard dose of x-rays, the anaphylactic antibodies anchored in the x-rayed area may be sufficient in amount to protect that area for a long period or perhaps even indefinitely. The succession of conditions is then inflammation—healing—(Table I, sensitized group).

The explanation which we have given obviously only answers the question why the ordinary process of an x-ray action on tissues should be altered when the organism is treated with serum parenterally; how this alteration is produced we cannot say because it is not known how either the x-rays or the foreign protein exert their effects upon the tissue cells.

The results reported in this paper emphasize a precaution which ought to be observed in all animal experimentation. Since mere sensitization with an alien protein alters the reactivity of an organism not only towards the specific alien protein itself, but also towards an unknown number of other, unrelated substances or even physical agents, it is obvious that sensitized animals cannot serve as normal controls until it has been demonstrated that both the sensitized and normal animals react to the same agent in the same manner and to the same degree. Discarded animals which have been subjected experimentally to the action of undenatured proteins of bacterial, protozoan, metazoan, or vegetable origin should be used in identified groups when they are reemployed for an investigation. Failure to respect this precaution perhaps explains some of the discordant results obtained in diverse studies of the same problem. It is further possible that some of the erratic fluctuations in the degree of a reaction observed in a group of supposedly normal animals have their cause in an unsuspected proteinization of the abnormal reactors. The possibility or even probability of unwittingly employing proteinized mammalian material cannot be denied, for most investigators are compelled to rely upon dealers for their animal stock. In com-

pensation for this uncertainty, we may perhaps look upon the abnormal reactors among a group of animals as indicators of a possible proteinized state, and thus gain a working hypothesis which may add to our knowledge of non-specific phenomena.

SUMMARY.

When a fixed area of the ears of rabbits is subjected to the action of a standard destructive dose of x-rays (30 skin units) the type of reaction resulting depends upon the previous treatment of the rabbit. (1) In normal rabbits a mild acute inflammation develops in the x-rayed area which leads at once to a perforating gangrene within an average of 15 days. (2) If rabbits are x-rayed and about 2 weeks later injected with horse serum for the first time, a mild acute inflammation appears which heals for a time; then a second, subacute inflammation sets in which leads to a perforating gangrene. The average time of the process from the first inflammation to gangrene is 32 days. (3) If rabbits are sensitized with horse serum and 10 days later are exposed locally to the standard dose of x-rays, the ensuing ear reaction is either similar to the second reaction described above, except that it may last up to 110 days, or the first inflammation leads to a healing which may be apparently permanent (340 + days). (4) If rabbits are first sensitized with horse serum, exposed locally to the standard dose of x-rays 10 days later, and 13 days after the x-ray treatment reinjected with horse serum, the reaction of the x-rayed area of the ears is in general similar to the second reaction described above (inflammation—healing—inflammation—gangrene). The average time of the whole process is about 42 days.

On the basis of the general hypothesis that an anaphylactic reaction is initiated in the body when the specific antibody meets its antigen, and that both antibody and antigen are rendered more or less functionally inert by their interaction, the following inferences may be drawn from our experimental results. (1) The protection from the effects of a standard destructive dose of x-rays which a previous sensitization confers, is referable to the presence of anaphylactic antibodies in the x-rayed area. (2) This protection is largely due to the anaphylactic antibodies which are anchored in the x-rayed area, and not to those which are free in the circulation. (3) An anaphylactic reaction

renders the anchored anaphylactic antibodies largely impotent as protective factors against the standard destructive x-ray dose, even though sensitization preceded exposure to the x-rays.   (4) An area treated with the standard destructive dose of x-rays is unable to produce or to anchor a sufficient amount of anaphylactic antibodies for protection from necrosis, when the x-ray treatment precedes the sensitization, or when the locally anchored anaphylactic antibodies are rendered functionally inactive by a general anaphylactic reaction.

It is possible that the procedure of increasing the resistance of the skin to a destructive dose of x-rays by means of a previous sensitization with protein may be applicable in the treatment of certain types of inoperable disease, when it is important to use massive doses of x-rays.

Animals which have been sensitized, or sensitized and reinjected with any undenatured alien protein, should not be reemployed as normal controls in any investigation unless trial has shown that these proteinized animals react quantitatively and qualitatively like normal animals.

The presence of an abnormal reactor in a group of supposedly normal animals may be an indication of a previous proteinization.

EXPLANATION OF PLATES.

The photographs of the rabbit ears were taken by transillumination while the blood vessels were in a dilated state.   The time of photographing was 181 days after x-raying.   The vessel traversing the middle of the ear is the central artery and in most figures its bifurcation near the upper pole of the ear can be seen. The marginal ear vein is also usually clearly visible.   The x-rayed area of the right ear is shown as a bald quadrilateral space.   Unfortunately the normal control rabbits were not photographed.

PLATE 100.

FIG. 1. Serum control group; Rabbit CX 16.   Perforating gangrene occurred about 50 days after x-ray treatment.

FIG. 2. Serum control group; Rabbit CX 17.   Perforating gangrene occurred about 50 days after x-ray treatment, but the process exceptionally is not yet complete although 181 days had passed since the x-raying.   This is shown by the slowly healing, superficial ulcer to the right of the perforation, appearing as a black patch in the photograph.

PLATE 101.

FIG. 3. Sensitized group; Rabbit HSX 12. This figure shows that the bald, x-rayed surface is perfectly smooth with no perforation or crusts. The central artery in the x-rayed area is markedly narrowed. This area has remained in the same state for over 340 days after the date of x-ray treatment.

FIG. 4. Sensitized group; Rabbit HSX 13. The x-rayed area is intact and bald and the central artery shows clearly a partial stenosis. The small black spot represents a slight hemorrhage from a collection of fine, tortuous, superficial vessels at that point. The x-rayed area has remained in this condition now for more than 340 days.

PLATE 102.

FIG. 5. Sensitized and reinjected group; Rabbit HSX 5. The perforating gangrene took place 85 days after x-ray treatment. This figure shows clearly how both the central artery and the marginal ear vein are narrowed in the x-rayed field.

FIG. 6. Sensitized and reinjected group; Rabbit HSX 7. Perforating gangrene took place 62 days after x-ray treatment. The partial stenosis of a vein in the x-rayed area is shown to the right of the perforation.

FIG 1.

FIG. 2.

Fig. 3.

Fig. 4.

(Auer and Witherbee: Reaction of normal skin to x-rays.)

FIG. 5.

FIG. 6.

(Auer and Witherbee: Reaction of normal skin to x-rays.)

ON THE PREPARATION OF GALACTONIC LACTONE.

By P. A. LEVENE and G. M. MEYER.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

The process of preparation of galactonic lactone is still associated with many difficulties. When the calcium salt of galactonic acid is suspended in water and treated with an equivalent weight of oxalic acid, the filtrate on concentration forms a crystalline deposit which is neither the pure acid nor its lactone, but is a complex mixture of several forms of the acid and of several forms of the lactone. A detailed analysis of the crystalline material was made by Nef.[1]

Pure monomolecular form of lactone is prepared in the following way. The calcium salt is suspended in water, to which an equivalent weight of oxalic acid is added. The filtrate is concentrated under diminished pressure until a crystalline deposit begins to form in the flask. The deposit is redissolved by warming and the syrupy liquid is poured into glacial acetic acid.

The substance which crystallizes out is the hydrated form of the lactone. It analyzed as follows:

0.1099 gm. of air-dried substance lost, on drying under dimished pressure at temperature of water vapor, 0.0114 gm.

0.0985 gm. of the air-dried substance gave on combustion 0.0482 gm. of H_2O and 0.1464 gm. of CO_2.

	Calculated for $(C_6H_{10}O_6 + H_2O)$. per cent	Found. per cent
H_2O	10.9	10.38

	Calculated for $(C_6H_{10}O_6)$. per cent	
C	40.40	40.53
H	5.62	5.47

---

[1] Nef, J. U., *Ann. Chem.*, 1914, cdiii, 273.

The practically anhydrous form is obtained by a single recrystallization of the original product from 99.5 per cent alcohol. This air-dried substance, on further drying under diminished pressure at the temperature of water vapor, lost but 2 per cent in weight. On titration with 0.1 N alkali, the fresh solution reacts alkaline to phenolphthalein after the addition of the first drop of alkali. When an excess of alkali is added the solution allowed to stand over night, and then titrated back with 0.1 N acid, 0.1000 gm. of the dry substance consumes 5.4 cc. of 0.1 N alkali. The theory requires 5.62 cc. of 0.1 N alkali.

This substance melts at 112° and gave the following optical rotation in water:

$$[\alpha]_D^{20} = \frac{-0.79 \times 100}{1 \times 1.0827} = -73.0°$$

after 96 hours

$$[\alpha]_D^{20} = \frac{-0.76 \times 100}{1 \times 1.0827} = -70.2°$$

after 16 days

$$[\alpha]_D^{20} = \frac{-0.69 \times 100}{1 \times 1.0827} = -63.7°$$

LECITHIN.

IV. LECITHIN OF THE BRAIN.

BY P. A. LEVENE AND IDA P. ROLF.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

It may seem unwarranted to reopen the question of brain lecithin. Does brain contain lecithin? Does this, if present, differ from lecithins of other organs? These questions at this date seem peculiar when one takes into consideration that Gobley,[1] who discovered lecithin in the egg yolk in 1846, reported its isolation from the brain in 1847. Later workers, still of the group of pioneers, substantiated these early observations. Among them the affirmative work of Thudichum[2] carries most weight since no other single worker has devoted as much energy and care to the isolation of the brain components.

In recent years, Koch[3] has claimed to have isolated lecithin from the brain tissue. On the other hand, Fränkel and Linnert[4] deny the presence of lecithin in the brain tissue of man. True, these authors are inclined to see in their finding a point of differentiation in the chemical structure of the human brain from that of other species. On the other hand, older workers never recorded any differences in the lipoids of the brain derived from different species, and the question naturally arose, does the negative finding of Fränkel and Linnert apply also to the brain of other species than of man? In the days of Gobley, and even in those of Thudichum, the methods of identification of individual lipoids were very imperfect and, *a priori*,

---

[1] Gobley, M., J. *pharm. chim.*, 1847, xi, 409; xii, 1.

[2] Thudichum, J. L. W., A treatise on the chemical constitution of the brain, London, 1884.

[3] Koch, W., Z. *physiol. Chem.*, 1902, xxxvi, 134.

[4] Fränkel, S., and Linnert, K., *Biochem. Z.*, 1910, xxiv, 268; xxvi, 44.

one would naturally be inclined to attribute more weight to results obtained by more recent work.

But, even granting the existence of brain lecithin, one is still in the dark on the question of its structure, relying only on the experimental data of the older workers. Very recently, and only in this laboratory were hydrolyses made on lecithins which did not contain considerable admixtures of cephalin. This work has brought out the fact that lecithins differ in the nature of the fatty acids entering into their structure. Thus it was found that while the unsaturated acid of the egg yolk was oleic,[5] that of the liver[6] was of the linolic series. As to the saturated acids, the lecithin of the egg yolk contained two acids, palmitic and stearic, while that of the liver seemed to contain only stearic. In view of our latest experience on the egg lecithin, this latter point concerning the liver lecithin is in need of reinvestigation. At any rate, the work on the egg lecithin and that on the liver lecithin suggest that there may exist a difference between individual lecithins. Thus the present work was directed towards the solution of two questions. First, of the existence of lecithin in the brain tissue, and, as this has been answered in the affirmative, there arose the second question as to the nature of its fatty acids.

As regards the preparation of lecithin from brain tissue, our experience is in accord with that of Thudichum. This author writes: "Lecithin . . . . is only with difficulty evolved from the brain, on account not only of the many stages of the processes necessary for its isolation, but also on account of its readiness to decompose under certain conditions." Early in our work, we obtained evidence of the presence of lecithin in the brain tissue. However, analytically pure lecithin was isolated only after a process of purification was evolved in the course of the work on the unsaturated lipoids of the liver.

This method is based on the observation that some impurities (cerebrosides) are insoluble in cold glacial acetic acid, others in a mixture of a glacial acetic and alcohol, whereas lecithin is soluble in both reagents. Hence, the crude material is dissolved in warm glacial acetic acid and the solution is allowed to cool. On cooling

[5] Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1921, xlvi, 193.

[6] Levene, P. A., and Ingvaldsen, T., *J. Biol. Chem.*, 1920, xliii, 359.

there is formed a precipitate consisting chiefly of cerebrosides. To the filtrate from this material ten volumes of 95 per cent alcohol are added. This treatment brings down a precipitate consisting mainly of cephalin. The filtrate from this second precipitate yields a product consisting of nearly equal proportions of lecithin and cephalin. Thus a sample consisting of 55 per cent of lecithin and 45 per cent of cephalin had the following elementary composition:

	per cent
C	65.87
H	10.45
N	1.91
P	3.89

For convenience of analysis and purification this sample was hydrogenated by Paal's method. It then had the following composition:

	per cent
C	65.69
H	10.94
N	1.92
P	3.79

$$\text{Ratio} \frac{NH_2N}{\text{Total N}} = \frac{38}{100}$$

This sample has approximately the same composition as the corresponding material prepared from the egg yolk. This finding is important in its bearing on the composition of brain cephalin. A brain cephalin having the elementary composition required by the theory is thus indicated and, it is hoped, will soon be prepared.

For the separation of lecithin from cephalin one still has to resort to its cadmium chloride salt. Again in preparing lecithin from the brain as from the egg yolk, one obtains a pure product more readily from the acetone-soluble fraction. The analysis of a sample of dihydrolecithin prepared from this fraction is reported in the experimental part of this paper. It had an elementary composition required by theory, and in other properties was identical with hydrolecithin prepared from egg yolk. Thus the existence of lecithin among the lipoids of brain tissue may be regarded as definitely established.

Regarding the chemical relationship of the brain lecithin to other lecithins, it was found that it had the same composition as lecithin from the egg yolk. The distinction between lecithins lies apparently in the differences of the character of their fatty acids. The fatty acids isolated from the egg lecithin are oleic, palmitic, and stearic acids. The same acids were also isolated from the brain lecithin. This finding suggests the possibility of the existence in the brain tissue, also, of more than one lecithin.

<div align="center">EXPERIMENTAL.</div>

1. Isolation of Lecithin from the Ethereal Extract of Ox Brains.

Ethereal extracts of desiccated ox brain tissue were used as the source of lecithin. The desiccated tissue was extracted with acetone prior to its extraction with ether containing 5 per cent of water. After concentration, the residual ethereal extract was poured into acetone. The resulting precipitate was redissolved in ether and allowed to stand over night at 10°C. Generally a sediment of "white matter" formed, which was removed by centrifugalization. The ethereal solution was again concentrated and precipitated by acetone. This operation was repeated until the ethereal solution on standing over night no longer gave a white precipitate. The ethereal solution was precipitated with cold alcohol and the precipitate was again dissolved in ether and again precipitated from alcohol. After a third such precipitation the combined alcoholic solutions were concentrated to a small bulk under diminished pressure, the solution being kept below room temperature during the process. The residual material was precipitated from acetone. From 200 ox brains 400 gm. of this lecithin fraction were obtained, having at this stage the following composition:

No. 282.   2 gm. of substance were hydrolyzed with 10 per cent HCl, neutralized, and concentrated to 25 cc.

5 cc. of this solution for Kjeldahl determination required 2.31 cc. of 0.1 N HCl, equivalent to 0.003234 gm. of nitrogen.

2 cc. of this solution for Van Slyke determination gave 1.04 cc. of nitrogen at $T = 23°$ and $P = 757$ mm., equivalent to 0.0005819 gm. of nitrogen.

$$\text{Ratio} \frac{\text{NH}_2\text{N}}{\text{Total N}} = \frac{44}{100}$$

0.1958 gm. of substance used for Kjeldahl determination required 2.70 cc. of 0.1 N HCl, equivalent to 0.0378 gm. of nitrogen.

0.2937 gm. of substance gave 0.0296 gm. of $Mg_2P_2O_7$.

0.1030 gm. of substance, dried under diminished pressure at temperature of water vapor gave on combustion 0.0992 gm. of H_2O, 0.2518 gm. of CO_2, and 0.0082 gm. of ash.

	Calculated for $C_{41}H_{78}O_8NP$ (cephalin).	Calculated for $C_{44}H_{86}O_9NP$ (lecithin).	Found No. 282 (calculated ash-free).
	per cent	per cent	per cent
C	66.17	65.26	67.70
H	10.57	10.95	11.07
N	1.88	1.77	1.93
P	4.17	3.92	2.79

100 gm. of No. 282 were dissolved with very gentle warming in 500 cc. of glacial acetic acid. This solution, on standing over night in the refrigerator, deposited a fine white precipitate (Precipitate I), consisting apparently of cerebrosides which had not been entirely removed by the previous treatment. The insoluble precipitates from two distinct lecithin fractions, both of which were isolated by the same method, amounted, respectively, to 10 and 15 per cent of the lecithin fraction from which they were extracted.

The mother liquor of this precipitated material was poured into ten volumes of 95 per cent alcohol. After standing over night in the refrigerator, a fine, flocculent, light-colored precipitate was formed (Precipitate II). On exposure to air, it coalesced to a sticky, dark-colored, gummy mass. In two experiments, this fraction consisted, respectively, of 8 and 6 per cent of the weight of the starting material.

The alcoholic solution was concentrated under diminished pressure, without heat, to dryness. This semisolid material (Precipitate III) was emulsified with water, and precipitated with acetone. 100 gm. of the original material yielded 65 gm. of a golden yellow, plastic, buttery mass, which showed no tendency to darken on exposure to air, and even after prolonged drying under diminished pressure did not become either hard or brittle.

The analytical data of Precipitates I, II, and III, follow:

Precipitate I.

No. 290. 2 gm. of substance were hydrolyzed with 10 per cent HCl, neutralized, and concentrated to 25 cc.

5 cc. of this solution for Kjeldahl determination required 28 cc. of 0.1 N HCl, equivalent to 0.000392 gm. of nitrogen.

2 cc. of this solution for Van Slyke determination gave 0.11 cc. of nitrogen at $T = 25°$ and $P = 752$ mm., equivalent to 0.0000603 gm. of nitrogen.

$$\text{Ratio} \frac{\text{NH}_2\text{N}}{\text{Total N}} = \frac{40}{100}$$

0.1500 gm. of substance containing 3.43 per cent moisture used for Kjeldahl determination required 1.62 cc. of 0.1 N HCl.

It contained no phosphorus.

0.1012 gm. of substance, dried under diminished pressure at temperature of water vapor gave on combustion 0.1030 gm. of H_2O, 0.2722 gm. of CO_2, and 0.0012 gm. of ash.

	Found No. 290. *per cent*
C.	73.34
H.	11.38
N.	1.17
P.	0.00

Precipitate II.

No. 291.   2 gm. of substance were hydrolyzed with 10 per cent HCl, neutralized, and concentrated to 25 cc.

5 cc. of this solution for Kjeldahl determination required 2.46 cc. of 0.1 N HCl, equivalent to 0.003444 gm. of nitrogen.

2 cc. of this solution for Van Slyke determination gave 2.06 cc. of N at $T = 25°$ and $P = 252$ mm., equivalent to 0.00113 gm. of nitrogen.

$$\text{Ratio} \frac{\text{NH}_2\text{N}}{\text{Total N}} = \frac{82}{100}$$

0.1939 gm. of substance used for Kjeldahl determination required 1.96 cc. of 0.1 N HCl.

0.2909 gm. of substance gave 0.0394 gm. of $Mg_2P_2O_7$.

0.1010 gm. of substance dried under diminished pressure at temperature of water vapor gave on combustion 0.0902 gm. of H_2O, 0.2388 gm. of CO_2, and 0.0122 gm. of ash.

	Calculated for $C_{41}H_{78}O_8NP$ (cephalin). *per cent*	Calculated for $C_{43}H_{86}O_9NP$ (lecithin). *per cent*	Found No. 291 (calculated ash- free). *per cent*
C.	66.17	65.26	65.80
H.	10.57	10.95	10.37
N.	1.88	1.77	1.41
P.	4.17	3.92	3.77

Precipitate III.

No. 247.   2 gm. of substance were hydrolyzed with 10 per cent HCl, neutralized, and concentrated to 25 cc.

5 cc. of this solution for Kjeldahl determination required 1.91 cc. of 0.1 N HCl, equivalent to 0.00267 gm. of nitrogen.

2 cc. of this solution for Van Slyke determination gave 0.85 cc. of N at $T = 21°$ and $P = 764.2$ mm., equivalent to 0.00048 gm. of nitrogen.

$$\text{Ratio} \frac{\text{NH}_2\text{N}}{\text{Total N}} = \frac{45}{100}$$

0.1943 gm. of substance used for Kjeldahl determination required 2.83 cc. of 0.1 N HCl.

0.2914 gm. of substance gave 0.0388 gm. of $Mg_2P_2O_7$.

0.1082 gm. of substance dried under diminished pressure at temperature of water vapor gave on combustion 0.0994 gm. of H_2O, 0.2570 gm. of CO_2, and 0.0116 gm. of ash.

No. 303.   2 gm. of substance were hydrolyzed with 10 per cent HCl, neutralized, and concentrated to 25 cc.

5 cc. of this solution for Kjeldahl determination required 3.26 cc. of 0.1 N HCl, equivalent to 0.003164 gm. of nitrogen.

2 cc. of this solution for Van Slyke determination gave 1.04 cc. of N at $T = 26°$, and $P = 742.8$ mm., equivalent to 0.00056 gm. of nitrogen.

$$\text{Ratio} \frac{\text{NH}_2\text{N}}{\text{Total N}} = \frac{46}{100}$$

0.1947 gm. of substance used for Kjeldahl determination required 2.66 cc. of 0.1 N HCl.

0.2921 gm. of substance gave 0.0408 gm. of $Mg_2P_2O_7$.

0.1078 gm. of substance dried under diminished pressure at temperature of water vapor gave on combustion 0.1007 gm. of H_2O, 0.2603 gm. of CO_2, and 0.0094 gm. of ash.

	Calculated for $C_{41}H_{78}O_8NP$ (cephalin).	Calculated for $C_{44}H_{80}O_9NP$ (lecithin).	Found No. 247 (calculated ash-free).	Found No. 303 (calculated ash-free).
	per cent	*per cent*	*per cent*	*per cent*
C................	66.17	65.26	66.25	65.87
H................	10.57	10.95	10.53	10.45
N................	1.88	1.77	2.04	1.91
P................	4.17	3.92	3.80	3.89

On reduction by Paal's method, rapid absorption of hydrogen occurred, and the dihydrolecithin crystallized on cooling.   10 gm. of No. 247 on hydrogenation gave 5 gm. of dihydrolecithin after re-

crystallization from methyl ethyl ketone. This substance gave the following rotation and analysis:

$$[\alpha]_D^{20} = \frac{+\,0.20 \times 100}{1 \times 4} = +\,5.00°$$

No. 244. 2 gm. of substance were hydrolyzed with 10 per cent HCl, neutralized, and concentrated to 25 cc.

5 cc. of this solution for Kjeldahl determination required 2.66 cc. of 0.1 N HCl, equivalent to 0.003724 gm. of nitrogen.

2 cc. of this solution for Van Slyke determination gave 1.02 cc. of N at $T = 23°$, and $P = 755$ mm., equivalent to 0.000569 gm. of nitrogen.

$$\text{Ratio } \frac{NH_2N}{\text{Total N}} = \frac{38}{100}$$

0.1932 gm. of substance used for Kjeldahl determination required 2.70 cc. of 0.1 N HCl.

0.2898 gm. of substance gave 0.0396 gm. of $Mg_2P_2O_7$.

0.1026 gm. of substance dried under diminished pressure at temperature of xylene vapor gave on combustion 0.0988 gm. of H_2O, 0.2436 gm. of CO_2, and 0.0104 gm. of ash.

	Calculated for $C_{41}H_{82}O_8NP$ (hydrocephalin). *per cent*	Calculated for $C_{43}H_{86}O_9NP$ (hydrolecithin). *per cent*	Found No. 244 (calculated ash-free). *per cent*
C...............................	65.81	65.10	65.69
H...............................	11.05	11.18	10.94
N...............................	1.87	1.77	1.92
P...............................	4.15	3.91	3.79

2. *Isolation of Lecithin from the Acetone Extract of Ox Brains.*

Acetone-Soluble Fraction.—The acetone-soluble material from ox brains, a semisolid mass consisting principally of cholesterol with a small admixture of lecithin and cephalin, was repeatedly extracted with large quantities of warm alcohol until the alcoholic solution after concentration gave no precipitate when treated with cadmium chloride solution. The alcoholic extract thus obtained was cooled to 0°C. and the cholesterol which crystallized on standing separated by filtration. The mother liquor was concentrated under diminished pressure to a smaller volume, and the cholesterol removed as completely as possible by crystallization. After filtration a saturated alcoholic solution of cadmium choloride was added to the filtrate

until the precipitation of the lecithin was complete. The precipitate was then washed by decantation with acetone, until it settled to the bottom of the jar in a finely divided white, easily filtered powder. The cadmium chloride salt of lecithin thus isolated contained, as indicated by a Van Slyke amino determination on the hydrolyzed material, from 30 to 40 per cent of cephalin.

This material was further purified by suspension in ether, adding water to the suspension until a clear solution was obtained, and reprecipitating the salt by pouring the solution into alcohol. If the material had not been sufficiently well washed with acetone after its original precipitation, filtration at this point would leave a rather gummy mass, the character of which could be markedly improved by using acetone instead of alcohol as a precipitant from the ether-water solution.

After three or four such precipitations from alcohol, the amino content usually fell to approximately 15 per cent. Its further purification was effected by dissolving the salt in toluene and precipitating it with ether.[5]

Several samples of material isolated in this way were combined and again precipitated by alcohol from an ether-water solution. About 125 gm. of material (No. 185) were obtained and analyzed as follows:

No. 185. 2 gm. of substance were hydrolyzed with HCl, neutralized, and concentrated to 25 cc.

5 cc. of this solution for Kjeldahl determination required 3.05 cc. of 0.1 N HCl, equivalent to 0.004270 gm. of nitrogen.

2 cc. of this solution for Van Slyke determination gave 0.1 cc. of nitrogen at $T = 23°$, and $P = 752$ mm., equivalent to 0.00005545 gm. of nitrogen.

$$\text{Ratio} \frac{NH_2N}{\text{Total N}} = \frac{3.2}{100}$$

This lecithin cadmium chloride was decomposed in 50 per cent alcoholic solution with ammonium carbonate in accordance with the method of Bergell.[7] Further purification was effected by emulsification with water, and subsequent precipitation by acetone (MacLean). It was then reduced by Paal's method, and the hydro-

[7] Bergell, P., *Ber. chem. Ges.*, 1900, xxxiii, 2584.

lecithin thus obtained, after one recrystallization from methyl ethyl ketone, gave the following analysis:

0.1443 gm. of substance used for Kjeldahl determination required 1.83 cc. of 0.1 N HCl.

0.2646 gm. of substance gave 0.0378 gm. of $Mg_2P_2O_7$.

0.1065 gm. of substance dried under diminished pressure at temperature of xylene vapor gave on combustion 0.1040 gm. of H_2O, 0.2561 gm. of CO_2, and 0.0105 gm. of ash.

	Calculated for $C_{49}H_{89}O_9NP$ (hydrolecithin). per cent	Found No. 188. per cent
C..	65.10	65.57
H..	11.18	10.92
N..	1.77	1.78
P..	3.91	3.98

Three observations of the optical rotation of this material were made in chloroform solutions of various concentrations. These were as follows:

$$[\alpha]_D^{20} = \frac{+0.23 \times 100}{2 \times 2} = +5.75°$$

$$[\alpha]_D^{20} = \frac{+0.33 \times 100}{1 \times 6} = +5.5°$$

$$[\alpha]_D^{20} = \frac{+0.30 \times 100}{0.5 \times 10} = +6.0°$$

3. Fatty Acids of Hydrolecithin.

By boiling 5 gm. of this material with ten parts of 10 per cent HCl for 8 hours, the fatty acids were isolated. The ether-soluble material from this hydrolysis mixture was precipitated by the addition of lead acetate. This salt, in benzene solution, was decomposed with hydrogen sulfide and the mixture of acids crystallizing from the concentrated benzene solution after two recrystallizations from respectively acetone and ethyl acetate, gave the following analysis:

0.0993 gm. of substance dried by fusion, gave on combustion 0.1125 gm. of H_2O and 0.2749 gm. of CO_2.

		Calculated for $C_{18}H_{36}O_2$. per cent	Found No. 197. per cent
C.......	....	75.99	75.50
H.......	....	12.70	12.67

The melting point of this material was lower than those of samples of Kahlbaum's "K" palmitic and stearic which had been purified for purposes of comparison. Melting point determinations of these three substances were carried out simultaneously. The following (corrected) melting points, the time interval averaging 7 seconds per degree, were obtained.

Palmitic acid. °C.	No. 197. °C.	Stearic acid. °C.
63–64	62–63	69–71

4. Fatty Acids of Lecithin Cadmium Chloride.

A. Saturated Fatty Acids.—A sample of amino-free lecithin cadmium chloride was boiled for 8 hours with methyl alcohol containing 5 per cent sulfuric acid. The esters of the saturated fatty acids crystallized from the hydrolysis mixture on cooling. After recrystallization they were fractionally distilled under a diminished pressure of 2.8 mm. These fractions were hydrolyzed and the elementary analyses, molecular weights as calculated from their titration values, and melting points of the respective acids are indicated below.

All samples of fatty acids were dried by fusion on a hot plate and to insure absolute freedom from moisture in the material used for elementary analysis were remelted under diminished pressure at the temperature of xylene vapor until constant weight was obtained. The molecular weights were calculated by the titration of approximately 1 gm. of acid dissolved in 10 cc. of toluene and 25 cc. of methyl alcohol (neutral to phenolphthalein) with 0.5 N NaOH, using phenolphthalein as an indicator.

The melting points as given are corrected and were taken at such a rate that the time interval per degree rise in temperature was 6 seconds.

First fraction, No. 185. B.P. = 166–168°C.
After hydrolysis to the acid:
0.0894 gm. of substance gave on combustion 0.1039 gm. of H_2O and 0.2460 gm. of CO_2.
1.0010 gm. of substance required for neutralization 7.70 cc. of 0.5 N NaOH corresponding to a molecular weight of 260.
M.P. = 61–62°C.

No. 185 was refractionated. The lower fraction was:
No. 192. B.P. = 156–159°C.

After hydrolysis to the acid:

0.1000 gm. of substance gave on combustion 0.1150 gm. of H_2O and 0.2754 gm. of CO_2.

0.8846 gm. of substance required for neutralization 6.9 cc. of 0.5 N NaOH corresponding to a molecular weight of 256.

M.P. = 63–64°C.

Second fraction, No. 186.   B.P. = 168–170°C.

After hydrolysis to the acid:

0.9602 gm. of substance required for neutralization 7.25 cc. of 0.5 N NaOH corresponding to a molecular weight of 265.

M.P. = 59–60°C.

Third fraction, No. 187.   B.P. = 170–180°C.

After hydrolysis to the acid:

1.0617 gm. of substance required for neutralization 7.70 cc. of 0.5 N NaOH corresponding to a molecular weight of 275.

M.P. = 59–60°C.

Fourth fraction, No. 188.   B.P. = 180–190°C.

After hydrolysis to the acid:

0.1006 gm. of substance gave on combustion 0.1162 gm. of H_2O and 0.2784 gm. of CO_2.

0.9566 gm. of substance required for neutralization 6.81 cc. of 0.5 N NaOH corresponding to a molecular weight of 281.

M.P. = 63–64°C.

Fifth fraction, No. 189.   B.P. = 190–200°C.

After hydrolysis to the acid:

0.1004 gm. of substance gave on combustion 0.1148 gm. of H_2O and 0.2794 gm. of CO_2.

0.8955 gm. of substance required for neutralization 6.30 cc. of 0.5 N NaOH. corresponding to a molecular weight of 284.

M.P. = 70–71°C.

Sample.	Boiling point of ester. $P = 2.8$ mm.	Analysis of acid.		Molecular weight of acid.	Melting point of acid.
		H	C		
	°C.	per cent	per cent		°C.
First fraction, No. 185..............	166–168	13.00	75.02	260	61–62
Refractionated lower fraction, No. 192..	156–159	12.87	75.10	256	63–64
Second fraction, No. 186.............	168–170			265	59–60
Third " " 187.............	170–180			275	59–60
Fourth " " 188.............	180–190	12.92	75.47	281	63–64
Fifth " " 189.............	190–200	12.79	75.89	284	70–71
Required for $C_{16}H_{32}O_2$ (palmitic acid)..		12.58	74.92	256	63–64
Required for $C_{18}H_{36}O_2$ (stearic acid)....		12.76	75.93	284	70–71

B. Unsaturated Fatty Acid.—The unsaturated ester was isolated from the mother liquor from which the saturated ester had crystallized, by extraction of the neutralized, concentrated residue with ether. Hydrolysis and subsequent decomposition of the sodium salt gave an acid with an iodine value of 88.

0.2446 gm. of substance absorbed 0.21664 gm. of iodine when titrated according to the method of Wijs.

	Calculated for $C_{18}H_{34}O_2$ (oleic acid).	Found No. 181.
Iodine number.................................	90	88

Reduction of this acid by Paal's method yielded an acid, the analysis of which corresponded to that of stearic acid.

No. 182.   0.1004 gm. of substance gave on combustion 0.1161 gm. of H_2O and 0.2810 gm. of CO_2.

1.3300 gm. of substance required for neutralization 9.40 cc. of 0.5 N NaOH corresponding to a molecular weight of 284.

	Analysis.		Molecular weight.	Melting point.
	H	C		
	per cent	*per cent*		°C.
No. 182......................................	12.93	76.32	284	70–71
Required for $C_{18}H_{36}O_2$ (stearic acid)	12.76	75.93	284	70–71

FIBRIN AND SERUM AS A CULTURE MEDIUM.

BY ALBERT H. EBELING, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

INTRODUCTION.

Various artificial media have been used so far in the cultivation of tissues. Lewis and Lewis used bouillon and agar,[1] salt solutions,[2] and other media of known chemical constitution,[3] but the growth was by no means equal to that in plasma or lymph, either in extent or duration. Swezy[4] obtained cell proliferation of chick embryo heart tissue in a medium composed of egg albumin and muscle extract. Carrel and Burrows[5] found homogenic as well as heterogenic serum a useful culture medium for embryonic chick tissue. Ingebrigtsen[6] studied the growth of tissue outside the organism in a medium composed of agar and serum. Smyth[7] devised a so called simplified medium composed of agar and trypsinized peptone solution for embryonic tissue culture. No attempt was made to undertake a comparative quantitative study of the amount of tissue produced in these different media. Burrows[8] pointed out that embryonic tissues grew as well in saline solution as in plasma. He thought that growth consisted in a spreading of the cells and not in any increase in the mass of tissue, and that it appeared to take place at the expense of the original fragment. It is certain that the tissues did not increase

[1] Lewis, M. R., and Lewis, W. H., *Bull. Johns Hopkins Hosp.*, 1911, xxii, 126.

[2] Lewis, M. R., *Anat. Rec.*, 1915–16, x, 287. Lewis, M. R., and Lewis, W. H., *J. Am. Med. Assn.*, 1911, lvi, 1795.

[3] Lewis, W. H., and Lewis, M. R., *Anat. Rec.*, 1912, vi, 207.

[4] Swezy, O., *Biol. Bull.*, 1915, xxviii, 47.

[5] Carrel, A., and Burrows, M. T., *J. Exp. Med.*, 1911, xiv, 244.

[6] Ingebrigtsen, R., *J. Exp. Med.*, 1912, xv, 397–398.

[7] Smyth, H. F., *J. Med. Research*, 1914–15, xxxi, 255.

[8] Burrows, M. T., *Tr. Cong. Am. Phys. and Surg.*, 1913, ix, 77.

in mass in any of the artificial media, nor could they be kept alive after a certain time. Therefore, these media could not be used for a quantitative study of the problem of growth.

It is known that the presence of embryo juice in adult plasma allows an indefinite growth of the fibroblasts and an increase in the mass of tissue. But the composition of this medium is complex and cannot be modified easily. It would be useful to find a medium endowed with the same properties as plasma and embryo juice, and more adaptable to the nature of the experiment.

The purpose of this article is to describe a technique for preparing a medium composed of fibrinogen, serum, and tissue juice, and to compare the growth of fibroblasts in this medium with that obtained in a medium composed of plasma and embryo juice.

<center>EXPERIMENTAL.</center>

The technique of Mellanby[9] was used in the preparation of fibrinogen. 10 cc. of normal adult chicken plasma were diluted with 90 cc. of sterile distilled water, and thoroughly shaken in an Erlenmeyer flask; 1 cc. of a 1 per cent acetic acid solution was added, drop by drop, and at the same time the mixture was agitated. The precipitate was allowed to settle partially in the cold for about 1 hour. The contents of the flask were then shaken and poured into centrifuge tubes, 25 cc. in each tube. After 10 minutes centrifugation, the supernatant fluid was decanted. The tubes were inverted over a sterile piece of filter paper for complete drainage. The precipitate contained in each centrifuge tube was combined and made up to 2.5 cc. with sterile, distilled water. When thoroughly mixed, it had the appearance of rich milk; on standing, a heavy sediment settled, superimposed by a layer of turbid fluid. Equal volumes of this suspension and Ringer's solution formed a slightly hazy, firm homogeneous clot after about 4 minutes. With an equal volume of serum, the mixture formed a clear fluid which did not coagulate after 10 minutes, but on the addition of a trace of embryonic tissue juice, coagulation occurred rapidly. The hydrogen ion concentration of such a preparation of fibrinogen was between 6 and 6.3. A mixture

[9] Mellanby, J., *J. Physiol.*, 1917, li, 396.

of 12.5 per cent fibrinogen suspension, 37.5 per cent chicken serum, and 50 per cent embryonic tissue juice, had a pH of between 7 and 7.3. Such a preparation coagulated in about 1 minute.

The experiments were begun with 48 hour cultures, derived from a strain of connective tissue in its 9th year *in vitro*.[10] Each fragment of tissue was divided in two parts and washed in Ringer's solution for about 40 seconds. One fragment was then cultivated in equal volumes of normal chicken plasma and embryonic tissue juice. The other fragment was cultivated in the experimental medium composed of one-fourth volume of fibrinogen suspension, three-fourths volume of chicken serum, and one volume of embryonic tissue juice. The constituents of this medium were first mixed by drawing them up

TABLE I.

Experiment No.	Culture No.	First passage.			Second passage.			Third passage.			Fourth passage.		
		Relative increase.		Ratio, $\frac{E}{C}$.	Relative increase.		Ratio, $\frac{E}{C}$.	Relative increase.		Ratio, $\frac{E}{C}$.	Relative increase.		Ratio, $\frac{E}{C}$.
		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.	
1	17555	17.5	16.2	0.93	12.5	11.6	0.93	10.3	9.2	0.89	11.0	8.9	0.81
											9.7	8.0	0.83
2	17536	21.5	18.3	0.85	21.7	19.3	0.88	10.5	8.0	0.76	10.0	9.2	0.92
					21.0	14.6	0.70	10.7	8.8	0.82	8.4	Liquefied.	

and expelling them from a bulb pipette, after they had been dropped into the hollow of a deep, concave slide. After 48 hours the tissues were washed and cultivated in the same medium.

The cultures were incubated at 39°C. Observations were made on the coagulation time, consistency, duration of the coagulum, and character of growth as compared with the controls. The cultures were traced, measured, and the relative increase was calculated, according to the technique mentioned in a previous article.[11]

The technique was developed in the course of thirty-five experiments. The results do not require detailed description because the

[10] Ebeling, A. H., *J. Exp. Med.*, 1919, xxx, 531.
[11] Ebeling, A. H., *J. Exp. Med.*, 1919, xxx, 533–534.

appearance of the coagulum and of the growth of the strain of fibroblasts was about the same as that of the control in plasma and embryo juice.   Generally, the width of the zone of new tissue and its density were slightly less in the experiment than in the control; the difference in most instances was approximately 10 per cent.   In other experiments the tissues were allowed to grow for 48 hours several times, and then transplanted into a medium of the same composition.   The results of two of these experiments are given in Table I and in Text-figs. 1 and 2.   In the table the figures for the relative growth of the tissue obtained in the control and experiment represent the amount
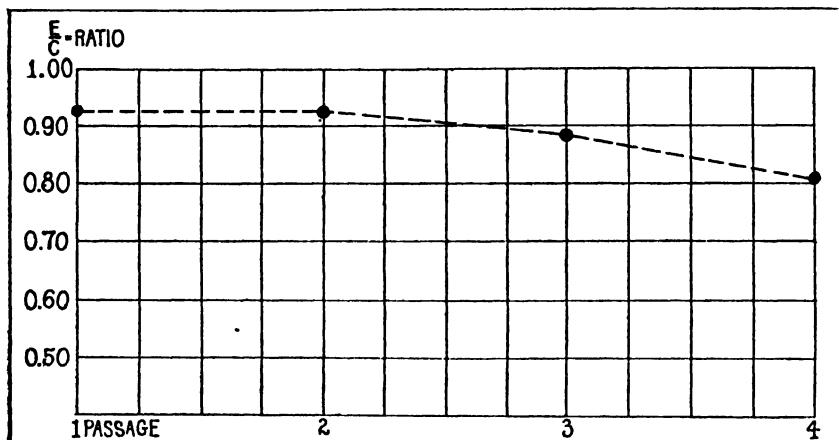
TEXT-FIG. 1. Experiment 1.   Ratio between the two relative growths, experiment and control.

of growth which was obtained during the time interval chosen (48 hours), expressed in function of the area of the primitive fragment of tissue.   The ratio between the two relative growths, $\dfrac{\text{Experiment}}{\text{Control}} = \dfrac{E}{C}$, is also given.   In Experiment 1 the tissues of both experiment and control were divided after the third passage.   In Experiment 2 the tissues were divided in the same way after the first passage.

There is a close relation between the relative growths of the experiments and of the controls.   This relation, expressed as ratios in the first experiment, was 0.93, 0.93, 0.89, 0.81, 0.83; and in the second,

0.85, 0.88, 0.70, 0.76, 0.82, 0.92. These figures express the fact that in the time elapsed between two passages, the amount of growth is very nearly the same both in the experiment and the control. A curve was plotted for both experiments in which these ratios were expressed in ordinates and the number of passages (48 hour intervals) in abscissæ.
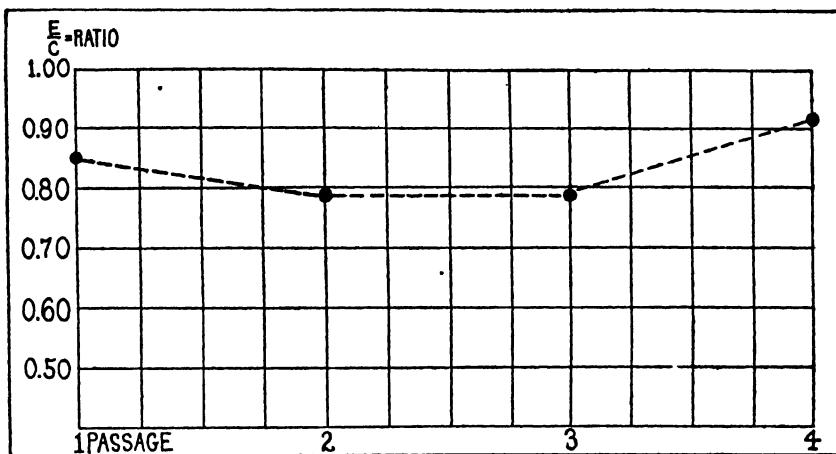
TEXT-FIG. 2. Experiment 2. Ratio between the two relative growths, experiment and control.

DISCUSSION.

A heavy precipitate could be obtained from 10 cc. of diluted plasma by adding 1.2 to 1.6 cc. of a 1 per cent acetic acid solution. But the final product was too acid. 1 cc. of the acetic acid solution produced a precipitate which, after suspension in distilled water, had a hydrogen ion concentration between 6 and 6.3. The hydrogen ion concentration of serum was from 8 to 8.3 and that of embryo juice from 7.3 to 7.5. When the precipitate suspension was dissolved in serum and mixed with embryo juice, the hydrogen ion concentration of the mixture varied from 7.3 to 7.5.

The coagulation was brought about by the addition of embryo juice. The addition of calcium was not essential to promote coagulation. On the contrary, a precipitate formed and liquefaction of

the coagulum occurred after 24 hours. The presence of serum is generally necessary to prevent liquefaction of the coagulum. In the experiments in which fibrinogen suspension was mixed with Ringer's solution or embryo juice alone, without serum, coagulation took place. But progressive liquefaction began soon afterwards. When serum was added in small quantities to fibrinogen suspension and embryo juice, the stability of the clot could be maintained.

The suspension of fibrinogen was used in various concentrations. If the medium contained more fibrinogen than plasma, the tissues did not grow well. When the concentration of fibrinogen was decreased, the coagulum was not dense enough and liquefaction often occurred within 24 hours.

It was found that excellent growth took place in a medium composed of 12.5 per cent fibrinogen suspension, 37.5 per cent serum, and 50 per cent embryo juice. Coagulation occurred within 1 minute and the coagulum was still firm after 48 hours.

The character of the growth of the old strain of connective tissue in the medium was not different from that observed in plasma. The growth was slightly less extensive in the experimental medium than in the control. It was easy to extirpate the fragment of tissue from its medium after 48 hours, and to transplant it into another medium. After every passage the amount of new tissue was about as large in the experiment as in the control. There was no doubt that the culture could have been kept alive for several more generations. This shows the possibility of keeping a strain of connective tissue in a medium composed of serum, fibrinogen, and embryo juice in about the same condition as in plasma and tissue juice.

CONCLUSIONS.

A technique is described by which a medium composed of fibrinogen suspension, serum, and embryo juice may be made.

Fibroblasts grew in this medium about as well as in plasma and embryo juice.

A strain of connective tissue in this medium remained practically as active as the control for several passages.

ENERGY AND VISION.

BY P. LECOMTE DU NOÜY.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Although a large number of papers and books have been published on the problems of vision (1), a very limited amount of work is to be found on the minimum energy necessary to produce visual sensation. The classical work of Langley (2) for different wave lengths the papers of Grijns and Noyons (3), Zwaardemaker (4), and Kries (5), for white light are always quoted, but the figures given by different authors do not always agree, discrepancies of 100 per cent, sometimes of 1000 per cent, being frequent, with no explanation. For this reason, it was thought advisable to check all these figures, in order to ascertain whence came the discrepancies. Furthermore, as Langley's figures are given by himself with a certain degree of approximation, and were calculated for the light emitted by the sun, we thought it would be interesting to check them by another method, for another source of light, the Nernst lamp, for instance. These are the reasons for carrying on this series of measurements.

In order to give an idea of how difficult it is to find a figure corresponding to the *minimum visibile* for a certain wave length, we will give an example. Langley's figures are quoted as follows for the wave length 0.55μ, for which the human eye shows a maximum of sensitivity:

By Broca (6) . . . . 5.6×10^{-9} ergs
By Henri and des Bancels (7) . . . . 3.0×10^{-8} ergs

whereas Langley's *real* figure, as given in his paper, is 2.8×10^{-9} ergs. Furthermore, Henri and des Bancels state on another page that 10^{-10} is the order of magnitude of the minimum energy necessary to produce the sensation of vision in the green (0.55μ), and Langley (2)[1] states that it is 1.0×10^{-8} (for practically the same radiation, 0.53μ). In order to clear this matter up, we have to go over Langley's paper carefully. Langley states and gives a solution for two different problems: first, determination of the intensity of light necessary to read a table

---

[1] Langley, (2), p. 23.

of logarithms or to discern any arbitrary character and second, determination of the *minimum visibile*; namely, the minimum of energy which can produce the sensation of light on the retina. The results of the first determinations are expressed in tables (2)[2] in function of the wave length and of the sensitiveness of the eye, in arbitrary figures related to the apparatus and inversely proportional to the energy. The results of the second determinations are expressed, on the contrary (2)[3] in the following way: reciprocals of calories = reciprocals of ergs (let us call this Table A), and these values stated in terms of horse-power (Table B), Now the meaning of these tables is quite ambiguous, and it is not surprising that authors have been mistaken in quoting them because, as they are given, they are only consistent provided the first table (A) is given in ergs per $\frac{1}{2}$ second. But as the figures in Table B, being expressed in horse-power, cannot be given in $\frac{1}{2}$ seconds (as the horse-power unit carries in itself its time unit and can only be used in connection with it, namely, 1 second), the figures of Table A must first be transformed into ergs per second, that is, multiplied by 2, to be identical with those of Table B. This is what has probably escaped the attention of Broca, and of Henri and des Bancels, and unfortunately, Broca took his figures from Table B, and Henri and des Bancels took theirs from Table A, so that all the figures of Broca are exactly double those of Henri and des Bancels. It is possible that these authors have not been mistaken, and that one of them (Broca) reduced the figures in Table A to ergs per second, whereas Henri and his coworker simply took them as they were. But it is most regrettable that none of them gave any indication as to the unit of time. Moreover, an important error is to be found in the figures of Henri and des Bancels due perhaps to misprint: for the wave length 0.55μ, they quote 3.0×10^{-8} ergs, (7)[4] instead of exactly 2.77×10^{-9}. If 3 may be taken as a roughly rounded figure for 2.77, however, the order of magnitude is different. In Broca's quotation, another error or misprint is also to be found: 3.6×10^{-3} ergs for 0.75μ, instead of 2.56×10^{-3}.

It may be of interest to compare the tables published by Broca, and Henri and des Bancels with the exact figures of Langley:

TABLE I.

	Langley.			Henri and des Bancels.	Broca.
	Reciprocal of				
				ergs	*ergs*
0.40μ	$1,500,000\frac{\text{ergs}}{\frac{1}{2}\text{sec.}}$ $= 6.7 \times 10^{-7}\frac{\text{ergs}}{\frac{1}{2}\text{sec.}}$	$= 1.33\times10^{-6}\frac{\text{ergs}}{\text{sec.}}$		6.7×10^{-7}	1.37×10^{-6}
0.55μ	$360,000,000$ " $= 2.77\times10^{-9}$ "	$= 5.55\times10^{-9}$ "		3.0×10^{-8}	5.6×10^{-9}
0.65μ	$1,600,000$ " $= 6.29\times10^{-7}$ "	$= 1.26\times10^{-6}$ "		6.0×10^{-7}	1.26×10^{-6}
0.75μ	780 " $= 1.23\times10^{-3}$ "	$= 2.56\times10^{-3}$ "		1.3×10^{-3}	3.6×10^{-3}

[2] Langley, (2), pp. 12, 13, 15.

[3] Langley, (2), p. 20.

[4] Henri and des Bancels, (7), p. 845.

Langley gives another series of figures (2)[1] by which he intends to express "the proportionate results for seven points in the normal spectrum, whose wave lengths correspond approximately with those of the ordinary color divisions, where unity is the amount of energy (about $\frac{1}{1000}$ erg) required to make us see light in the crimson of the spectrum near A." According to this definition, this scale corresponds to the *minimum visibile*.

Wave lengths......................	0.40	0.47	0.53	0.58	0.60	0.65	0.75
Luminosity (visual effect)...........	1.600	62.000	100.000	28.000	14.000	1.200	1

Expressed in negative powers of 10, in order to facilitate comparison, we have (unity being 10^{-3} ergs, no indication being given concerning the time):

Wave lengths........	0.40	0.53	0.65	0.75
Energy..............	6.2×10^{-7}	1×10^{-8}	8.3×10^{-7}	1×10^{-8}

The first figure (for 0.40μ) agrees well with that given by Langley (2)[3] in his other tables. The second one (0.53μ) does not agree at all, and the slight difference in wave length cannot be regarded as the cause of the discrepancy. The third one agrees within 25 per cent and the last one also, approximately. We see no explanation for this discrepancy, which cannot be due to a misprint.

Therefore, it was desirable to settle the question, since Langley's data are so misleading that good authors have made errors simply in quoting them. Quite recently, Joly (8) published a very interesting article on a quantum theory of vision, and although he does not share Henri's opinion on the subject, quotes one of his figures, 5×10^{-12} ergs for the threshold of sensitivity for white light. Now, we have tried in vain to find such a figure in two of the papers of Henri and des Bancels, as the indications of the source are missing. As far as we know, they did not make any measurements themselves, but simply quoted those of Grijns and Noyons. They quote the figures given by Grijns and Noyons, 4.4×10^{-11} ergs. Even if we admit that only 10 per cent of the energy is radiated under the form of light (9), we obtain 3.96×10^{-12}, and not 5×10^{-12}. It is regrettable that Professor Joly did not give the bibliographic reference.

Method.

An integration method was used. In other words, a curve representing the intensities of the dispersed beam after its passage through the prism was plotted in function of the wave lengths on coordinate paper. It is clear that the area delimited by this curve and certain

limits, arbitrarily chosen, that is to say, the integral of the curve between these limits, will express the total energy radiated. As the source yields in the same time invisible and visible rays, and as the methods used for measuring the radiation give us figures corresponding to the total radiation, R (visible $+$ invisible), a segment extending between the limits of the visible spectrum must also be integrated. This latter integration gives the quantity of energy spread in the visible part of the spectrum; let it be L. Then the ratio of these two areas will be the luminous efficiency of the source, and will be expressed by $\dfrac{L}{R} = E$. The percentage of the visible to the invisible is now known. Let us call I the intensities in function of the wave lengths, λ_1 the lower limit of integration, λ_2 the upper limit of integration for the visible, then:

$$E = \frac{L}{R} = \frac{\displaystyle\int_{\lambda_1}^{\lambda_2} I\,d\lambda}{\displaystyle\int_{0}^{\infty} I\,d\lambda}$$

The quantity of energy spread by the slit over the visible spectrum being thus known, a suitable screening of each monochromatic light decreases its intensity until the threshold of sensitivity is reached. Knowing exactly the amount of energy absorbed by the screens, the amount which is allowed to pass may be calculated easily: it is the minimum energy necessary to produce visual sensation.

Technique.

Limits of Integration.—Limits of Total Radiation.—Lower limit: For most light sources, the energy in the ultra-violet is so small that the lower limit, 0.4 μ, may be taken as zero without any appreciable error. Gage (10) takes it as the limit in his study of the electric arc, which is one of the richest sources in ultra-violet. The Nernst lamp, on the contrary, yields very little ultra-violet radiation, and it was assumed that this limit could safely be taken. Upper limit: The plotting of energy distribution curves showed that above 7μ in the infra-red, the amount of energy radiated by the Nernst

lamp was very small, as compared to that radiated beyond. From 7 μ to 10 μ, it amounts to less than 1 per cent of the total. As the other errors involved by the method are of a greater order of magnitude, it was adopted as the upper limit, for the total radiation.

Limit between the Red and the Infra-Red.—(Upper limit of integration of the visible spectrum). Langley, although he does not specify it, seems to have chosen 0.75 μ as the upper limit. Many workers have chosen 0.8 μ (as the eye is sensitive to the radiations up to 0.8 μ). Some have preferred 0.76 μ, others 0.7 μ. The reason for the importance of this determination is that energy increases very much between 0.7 μ and 0.8 μ, whereas the impression on the eye is very slightly changed. In other words, the shifting of the limit from 0.8 μ to 0.7 μ will change considerably the amount of energy spent in the visible spectrum, whereas the effect on the eye will hardly be noticeable, since it only brings in very faint, deep red rays which, if absent, do not modify one's impression appreciably. On the other hand, if it is sought to determine the minimum of energy necessary to make the red rays between 0.7 μ and 0.8 μ impress the retina, one has to shift the limit as high as 0.8 μ. And in this case, all the values given for the energy of radiations below 0.7 μ will be altered (by more than 27 per cent). Therefore, in this paper, the two figures are given, so that one may compare the results.

The study of luminosity curves shows that, by removing the part of the spectrum extending beyond 0.7 μ, the total luminosity is only decreased by 0.4 per cent. As König and Brodhun (11) have shown that the human eye was just able to detect a change in luminosity when it amounted to 1.6 per cent, we feel that this limit is advisable.

Measurement of Total Radiation.

The first step was to measure the value in absolute units of the total radiation of the Nernst lamp, with which it was intended to experiment; for it was difficult, owing to the discrepancies found in the figures given by different authors, Lux (12), Hartman (13), Ingersoll (14), etc., to rely upon data found in literature.

The source was an ordinary Nernst lamp, (110 volts, 1.3 amperes). In order to prevent any fluctuations due to cooling by air currents, the glower was enclosed in a brass chamber, with just one rectangular

slit (20 + 3 mm.), in front of the glower. The reflection from the heater and porcelain support was suppressed by fixing the glower by means of its platinum wires at the end of two leads. The inside of the chamber was blackened with soot. A voltmeter was placed across the terminals, and an ammeter in series, so as to know exactly the input in watts. Under normal conditions, it was found to be 87.5 volts × 1.05 amperes = 91.875 watts. These 91.875 watts are not all transmitted by radiation, but part of them are taken away by conduction and convection by the air. Lux and others give the ratio $\dfrac{\text{input}}{\text{radiation.}}$. But as these figures may correspond to differ-ent types of lamps, it was found safer to measure it directly. Besides, this would allow us, by a simple calculation, to check our radiation data against those published previously on the Nernst lamp.

It was first attempted to use a specially made mercury thermome-ter, with a known weight of mercury in a known weight of glass, blackened on the bulb of which the rays emerging from a 0.1 sq. cm. slit were concentrated by means of a fluorite lens of short focus. This process showed a lack of sensitivity and it was necessary to check it by means of an electric method. Although less difficult to handle than a bolometric device, the following apparatus required a great deal of care and time. A thermopile was made of copper and constantan wires, with ten elements, disposed linearly; the cold ends were simply bent out. On the top of the welded ends, carefully planed and ground, a thin piece of tin-foil exactly 1 mm. wide and 1 cm. long was applied and fixed with a very thin layer of shellac. Then the tin-foil was cut carefully between the welded ends, leaving a little square table of very nearly 1 sq. mm. on each thermocouple. These were blackened with soot, and the whole thermopile fixed in a thermostat. The rays were allowed to fall on the pile through an adjustable slit, and the distance between the source and the couples made equal to 1 meter. The method consisted in compen-sating the heat generated by the incoming radiation, by the current sent in a strip of constantan placed near the cold ends of the ther-mocouples, in a tiny calorimeter, 2 cc. in capacity, filled with oil, and well isolated. The following formula was used:

$$E = Ki^2 \frac{\text{cal. gr.}}{0.1 \text{ sq. cm. sec.}}$$

K being a constant (function of the resistance of the constantan strip) of the instrument, calculated and experimentally checked, equal to 0.21, we measured a current of 0.0028 amperes; this gives:

$$0.21 \times (0.0028)^2 \frac{\text{cal. gr.}}{\text{sec. 0.1 sq. cm.}} = 0.00000165$$

as 0.2388 cal. gr. = 1 watt sec., it corresponds to 0.000694 watts by sq. cm.

Correction for Equatorial Radiation.—This corresponds to the homogeneous radiation of a punctual source of energy of 87 watts; that is, it would require 87 watts from a punctual source to radiate spherically in all directions an amount of energy of that magnitude. We have measured this amount equatorially, that is, normally to a line normal to the glower itself, and, of course, in the best conditions of radiation. But as the beam of light assumes a greater deviation from the equatorial plane, in the case of an incandescent rod, in other words, as the square centimeter exposed to the rays stands higher in latitude on the sphere, the amount of energy radiated is decreased, since the rays are no longer emitted perpendicularly by the rod. Around the two poles, there is even a region where there is no radiation at all. The result is that, whereas the source acts as radiating 87 watts equatorially, it radiates much less as soon as we reach higher latitudes, and becomes zero at the poles, and the mean value of the radiation is much less than 87 watts. It is known that by multiplying the energy radiated equatorially by $\frac{\pi}{4}$, the real value of the radiating energy from the source is known. In this case,

$$87 \times \frac{\pi}{4} = 68.3 \text{ watts.}$$

Hence, out of the 92 watts sent into the filament, only 68.3 are radiated, and 23.7 are lost by convection and conduction. The ratio $\frac{92}{68.3} = 1.35$ is in excellent agreement with the figure given by Lux: 1.34.

This figure may be checked in another way: the input in the filament being 91.8 watts, roughly 92 watts, it will radiate equatorially $92 \times \frac{4}{\pi} = 118$ watts,

approximately. The quantity radiated actually as measured equatorially, being 87 watts, the ratio $\dfrac{118}{87} = 1.35$ gives the amount of energy lost.

Calculation of the Luminous Efficiency, and Corrections.—The next step was to determine the ratios

$$\frac{\displaystyle\int_{0.4}^{0.7} I d\lambda}{\displaystyle\int_{0.4}^{.7} I d\lambda} \quad \text{and} \quad \frac{\displaystyle\int_{0.4}^{0.8} I d\lambda}{\displaystyle\int_{0.4}^{.7} I d\lambda} = \frac{I}{R}$$

This ratio has been calculated by many authors, Lux, Nichols and Coblentz (15), Ingersoll, Ångström (16), Stewart and Hoxie (17), etc. Their methods were different and their results do not always check perfectly, (some varying by more than 50 per cent, for example, those of Lux and Ångström.) Some of the workers used methods based upon the absorption of one part of the spectrum by water cells in which different substances were dissolved, (copper sulphate, iodine). It has been shown by Nichols and Coblentz that none of these methods based on absorption were reliable. Ingersoll studied the Nernst lamp and published figures of observed luminous efficiency, which vary greatly according to different lamps, and besides, correspond to burners whose consumption was not that of our lamp, (89 watts). Therefore, the energy distribution curve of our burner was plotted by means of a Hilger Infra-Red Spectrometer.[5]

Correction for the Non-Normal Spectrum.—The spectrum was corrected for the lack of homogeneity of the dispersed beam. Indeed, the refracted rays are contracted in certain parts of the spectrum, and expanded in others, so that, for instance, the same slit opening (*e.g.*, 0.25 mm.) covers 0.015 μ on the spectrum at a mean wave length 0.68 μ (from 0.6725 μ to 0.6875 μ), and as much as 0.266 μ, more than 17 times as much, at the mean wave length 2.66 μ (from 2.53 μ to 2.79 μ). This correction was introduced by the consideration of the geometry of the screw motion, (pitch of screw in relation to

[5] Instrument No. 281, Rock salt prism, Angle 59°. 57'. 30".

rotation of the prism table), and the use of the dispersion formula, given by Paschen (18):

$$n^2 = a^2 + \frac{M_1}{\lambda^2 - \lambda_1^2} + \frac{M_2}{\lambda^2 - \lambda_2^2} - K\lambda^2 - h\lambda^4$$

Then, the range of the spectrum embraced by a given slit was checked by moving a spectrum line across the slit, and reading the result on the drum. The right edge of the D lines (sodium), for instance, was brought in contact with the right edge of the slit (0.25 mm. opening), and the reading made. Then it was moved toward the left until the whole D line just disappeared, and another reading made. The result was 0.008 μ. This was done for the lines of copper (0.4955 μ, 0.5292 μ), mercury (0.5461 μ), sodium (0.5893 μ), and cadmium (0.6439 μ). For the infra-red, the data are published by Hilger (19).

It was decided to take the area covered at 2.66 μ by a slit 0.025 mm. wide as unit, (0.026 μ) and to fix the slit in such a way, for every wave length, as to cover the same range. A high sensitivity Leeds and Northrup galvanometer was used in connection with the thermopile, (galvanometer resistance = 12 ohms).

Corrections of the Absorptions Due to the Spectrometer.—But, before integrating the plotted curve, another very important correction had to be introduced regarding the absorption by the golden mirrors, because the energy distribution curve does not correspond to the total amount received by the collimator slit, and because the absorption is much greater for short than for long wave lengths. Fig. 1 shows how the absorption varies for different wave lengths.

If R is the coefficient of reflection, and 3 the number of mirrors, the amount of energy reflected is expressed by

$$I = I_0 R^3$$

It is easily seen that for 0.5 μ, for example, only $\dfrac{1}{9.6}$ of the incident light is transmitted, and much less still for 0.4 μ.

The absorption by the prism amounts to very little. Theoretically, from the formula, $J = J_0 K^c$, where c is the length of the path of light in the rock salt and K a constant equal to 1 between 0 and 9 μ, it is equal to zero. We found that, practically, for the visible,
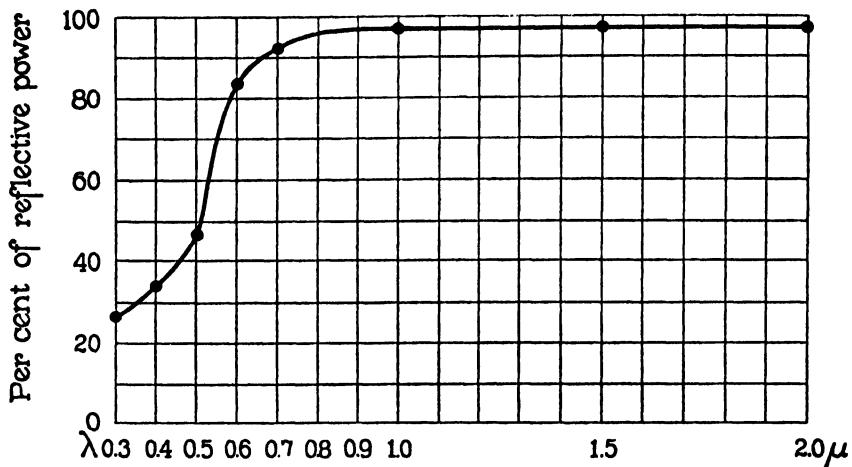
FIG. 1. Reflection of monochromatic light by gold.
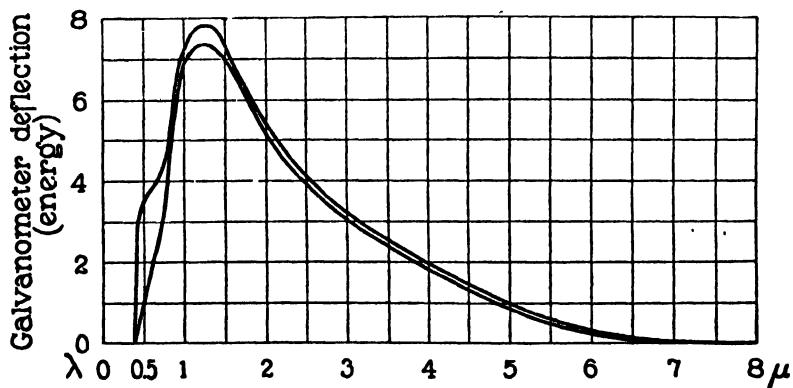
FIG. 2. Energy distribution curves for Nernst lamp, corrected and uncorrected for mirror absorption. Outside curve corrected; inside curve uncorrected.

it really amounts to 1.5 per cent approximately, by concentrating the beam of light before and after passing through the prism.

Finally, the curves were integrated graphically. Fig. 2 shows the different aspects of the curves before and after corrections due to absorption. (These curves are not to scale, in order to emphasize the difference.) The results were:

$$R = \int_{0.4}^{7} = 110.3, \qquad L_{0.8} = \int_{0.4}^{0.8} = 4.8, \qquad L_{0.7} = \int_{0.4}^{0.7} = 3.6$$

The ratios are:

$$\text{Upper limit} = 0.8, \qquad \frac{L}{R} = 0.0435$$

$$\text{Upper limit} = 0.7, \qquad \frac{L}{R} = 0.0316$$

These results are in good agreement with those of Ingersoll, who, with the upper limit 0.76, found figures between 0.036 and 0.046, and in contradiction with those of Drude (20), who gives 12 per cent as luminous efficiency of Nernst lamps, (instead of 4.35 per cent). Lux gives 5.96 per cent, but takes no account of the fact that the screening method he used allowed most of the radiation up to 1.2 μ to pass. Coblentz and Nichols found 0.033 for the efficiency of acetylene flame.

Hence, as we have established that our source radiated 87 watts equatorially, the quantity radiated as luminous waves is equal to 4.35 per cent of 87 (limit 0.8 μ) = 3.8 watts, and 3.16 per cent of 87 (limit 0.7 μ) = 2.75 watts. Let us take the quantity corresponding to the limit 0.7 μ for example. These 2.75 watts are radiated at the distance of 103 cm. from the source, (distance of the thermopile) by square centimeter. The energy then becomes 0.0000206 watts. The area of the slit being 0.1 sq. cm., it only receives 0.00000206 watt seconds, or 20.6 $\times$ 10^{-7} watts = 20.6 ergs. This quantity of energy is spread over the range delimited by the upper and lower limits of integration (0.4 μ to 0.7 μ) and the energy distribution curve corrected for the absorption by mirrors, and by the prism, that is, over a surface of 83 sq. cm., (obtained by graphical integration of the curve, Fig. 3). This corresponds, in the scale chosen, to 0.248 ergs by unit of surface. Similarly, between the limits 0.4 μ

and 0.8 μ, we find 0.343 ergs by unit of surface. In order to determine the efficient amount of energy which will affect the eye, it will easily be seen that this quantity will only have to be multiplied by the segment delimitated on the same chart, by the two ordinates corresponding to the range covered by the slit on the spectrum and the energy distribution curve uncorrected for mirror absorption.
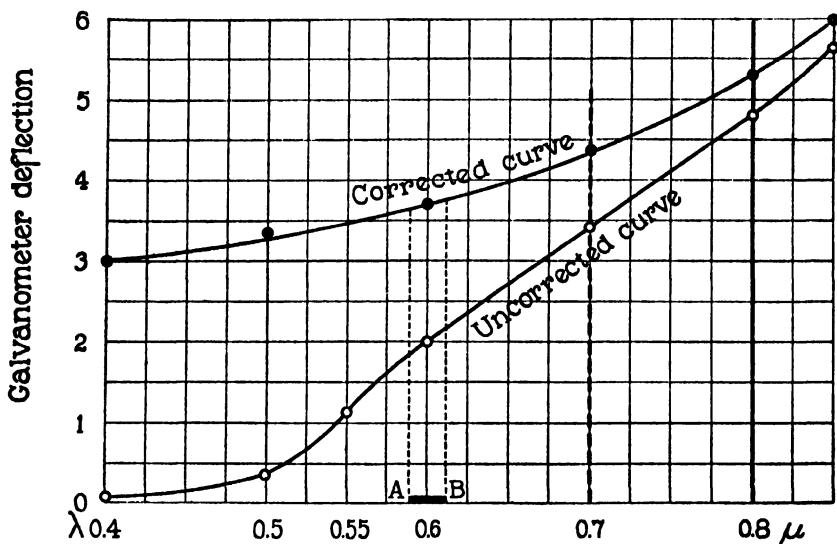
FIG. 3. Energy distribution curve, corrected and uncorrected, in a larger scale in the visible part of the spectrum. *A B* represents the area covered by a slit 0.25 mm. wide.

Test of the Eyes.

In order to reduce the intensity of light by a known quantity, a set of absorbing screens was prepared carefully. It was sought to look directly into the beam of light instead of using reflected light, in order to avoid the errors arising from the reflection of very faint radiations. By getting screens which could decrease by the same known amount, for example, 90 per cent, the intensity of the incident light, the simple formula

$$I = I_0 K$$

in which I = emergent light, I_0 = incident light, $K = \dfrac{I_0}{I}$ and n = the number of screens interposed, leads to this:

$$\text{Log } I_n = \text{Log } I - n \text{ Log } K$$

and as $K = 10$

$$\text{Log } I_n = \text{Log } I_0 - n$$

whence

$$I_n = I_0 \ 10^{-n}$$

The number of screens interposed will itself give the order of magnitude of the out-coming energy: 2 screens will mean that the energy is decreased by 100; 4 screens, by 10,000, etc.

It was found that especially prepared white paper fulfilled the requirements better than any other screen. Sheets of the same paper were chosen, (mean thickness 0.09 mm.), and placed exactly in front of the thermopile, then the throw of the galvanometer was observed; the paper was removed and another reading made at three different wave lengths. A great number were tested, and as we were unable to get ten sheets exactly similar, the thicker were placed on a plane surface and evenly rubbed down with very thin sand, then glossed again with a piece of round glass. They were frequently tested during this process, and finally the following results were considered as satisfactory. (The figures express the ratio $\dfrac{I}{I_0}$).

TABLE II.

Sheets.	0.55μ
No.	
1	0.100
2	0.103
3	0.103
4	0.095
5	0.0995
6	0.105
7	0.100
8	0.0965
Mean value....................	0.1005

This particular paper was less transparent for the extreme red than for the green, a fact which had to be taken into account.

Measurements.—Eighteen persons were examined; two series of experiments were performed: one after 8 minutes in the dark, and one after 25 minutes. Only five persons were examined for all wave lengths. The others were merely tested for the radiation 0.55. The measurements were carried on in the following way: When nine sheets of paper were placed exactly against the slit, generally no light could be seen. Then, one after the other, the sheets were removed, according to the intensity of light, and usually, owing to the relatively large area (0.1 sq. cm.), there was no difficulty whatever in determining the order of magnitude of the *minimum visibile*. Namely, one sheet added gave a black impression, and this sheet removed left a visible, although very faintly colored, image of the slit. We sought, as Langley did, to determine the *minimum visibile*, defining this to be, not the smallest light whose existence it is possible to suspect, or even to be reasonably certain of, but a light which is observed to vanish and reappear when silently occulted and restored by an assistant without the observer's knowledge (Fig. 4).

On top of the last sheet of paper, another slit was placed, across the first one. Its jaws were cut in such a way that a square opening was left between them, (see Fig. 5); thus a square or rectangular figure was delimited by four moving lines. At first, the slit was adjusted so as to cut a little window of 1 sq. mm. on the luminous spot. If the window could not be seen, the jaws of the slit were moved micrometrically until the spot became visible. The maximum opening corresponded to a vertical motion of 5 mm.

It was found that most women generally require more time than men to reach the same degree of sensitivity. Most of the men tested became adapted in 5 minutes (viz., could see a light corresponding to an energy of the order of magnitude 10^{-9}) while it required 15 to 20 minutes for women to see the same thing. Moreover, some of them could only see the spot spasmodically appearing and disappearing, while men had a continuous impression. Only an increase in the intensity of about 50 to 100 per cent was able to give them the same visual impression. As will be seen, the differences vary between 0 and 25 per cent among men for a given wave length;
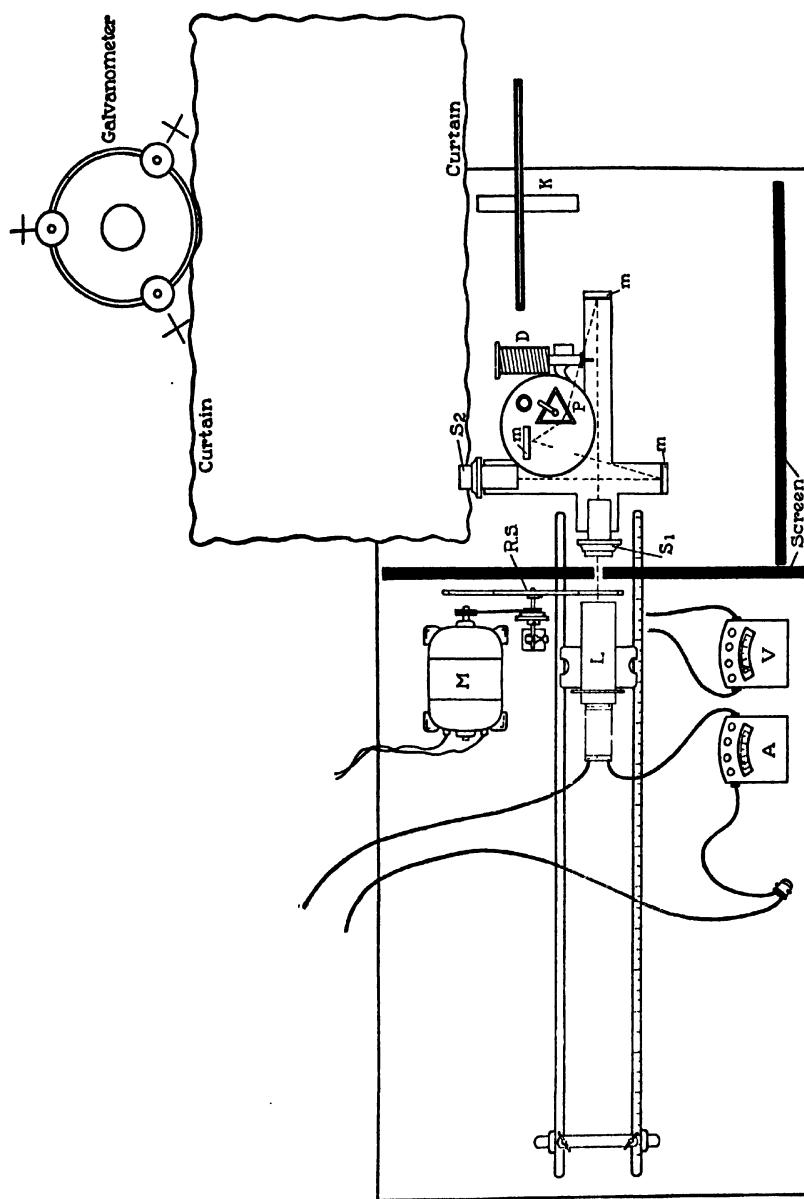
FIG. 4. Apparatus set for the test of the eyes. The observer was looking through slit S₂. L, Nernst lamp; M, motor; R. S. revolving shutter; A, ammeter; V, voltmeter; S₁, S₂, slits; P, rock salt prism; D, calibrated drum; m, m, mirrors; K, lamp and scale.

among women, between 0 and 100 per cent. One man showed a marked difference.

The figures express the energy necessary for continuous impression but by making the same assumption as Langley concerning the
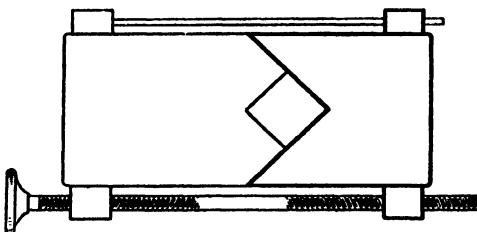
FIG. 5. Adjustable window.

minimum amount of time necessary to perceive distinctly a very faint light, (about ½ second), these figures may be expressed in ergs per ½ second, by dividing them by 2.

The size of the retinal image was approximately 0.01 sq. mm. ±0.002.

TABLE III.

Men.

Observer.	Age.		0.4 to 0.8 μ	0.4 to 0.7 μ
	yrs.		$\dfrac{ergs}{sec.}$	$\dfrac{ergs}{sec.}$
A	29	Normal sight.	7.1×10^{-9}	4.55×10^{-9}
B	36	" "	8.2×10^{-9}	5.9×10^{-9}
C	45	Short sighted.	7.1×10^{-9}	4.55×10^{-9}
D	36	" "	8.3×10^{-9}	5.9×10^{-9}
E	28	Normal sight.	8.3×10^{-9}	5.95×10^{-9}
F	23	" "	8.1×10^{-9}	5.95×10^{-9}
G	19	" "	8.6×10^{-9}	6.1×10^{-9}
H	30	" "	7.4×10^{-9}	5.25×10^{-9}
I*	27	" "	1.0×10^{-8}	8.6×10^{-9}
Mean values.............................			8.1×10^{-9}	5.85×10^{-9}

* Observer "I" was included in the mean, although he seemed to be quite out of the normal.

First Series of Experiments.—The observers were protected by a curtain from all light, and waited until their eyes had become quite sensitive before making the experiments. 8 minutes in absolute darkness seemed to be sufficient for men. These first figures will show the difference between the rapidity of adaptation of men and women. Wave length 0.55 μ (Tables III and IV).

TABLE IV.

Women.

Observer.	Age.		0.4 to 0.8 μ
	yrs.		$\dfrac{ergs}{sec.}$
J	30	Normal sight.	8×10^{-9}
K	30	Short sighted.	2×10^{-8}
L	25	" "	9×10^{-9}
M	22	Normal sight.	1×10^{-8}
N	24	" "	2×10^{-7}
O	23	" "	3×10^{-8}
P	19	" "	2×10^{-8}
Q	42	" "	4×10^{-8}
R	40	" "	5×10^{-8}

Obviously, for women the differences are so great that a mean value would have no significance at all.

It was found that it took over 20 minutes for observers J, M, and O to reach the same sensitivity as men, viz., less than 7×10^{-9}. As Langley does not give any precision concerning the time of adaptation, we may compare his figures to the mean value found for men: Langley (0.55 μ), $5.55 \times 10^{-9} \dfrac{ergs}{sec.}$. We found slightly larger figures:

$$\int_{0.4}^{0.7} \; : \; 5.85 \times 10^{-9} \frac{ergs}{sec.}$$

$$\int_{0.4}^{0.8} \; : \; 8.1 \times 10^{-9} \frac{ergs}{sec.}$$

But after more than 20 minutes in the dark, the eye becomes more sensitive still, and we obtained the following figures (Table V). These figures are smaller than those given by Langley, but as he did not state the length of time which the eyes of his experimenters

were kept in the dark, and as we have seen that the sensitivity is increased over 100 per cent by a stay of 25 minutes instead of 8 or 10, they cannot well be compared. Generally, at least, they are of the same order of magnitude for the wave length 0.55 μ. A stay of 1 hour in absolute darkness did not seem to increase the sensitivity beyond these figures.

It must be pointed out that the figures corresponding to the wave length 0.4 μ are doubtful, as the spectrometer which was used was not fit for the measurements in that part of the spectrum, owing to the gilded mirrors. They are only given as approximations. It must also be borne in mind that these quantities of energy do not

TABLE V.

25 Minutes in the Dark. $\int_{0.4}^{0.8}$

Observers.	0.4	0.5	0.55	0.65	0.68	0.72
(*Women*)						
J	2.5×10^{-6}	1.6×10^{-8}	3×10^{-9}	2×10^{-7}	2×10^{-6}	3×10^{-6}
O	5×10^{-6}	2.3×10^{-8}	4.5×10^{-9}	3.5×10^{-7}	5×10^{-6}	4×10^{-6}
(*Men*)						
B	5×10^{-7}	1.4×10^{-8}	2×10^{-9}	1.5×10^{-7}	1.9×10^{-6}	2×10^{-5}
D	5×10^{-7}	1.3×10^{-8}	2×10^{-9}	1.5×10^{-7}	2×10^{-6}	2×10^{-5}
F	8×10^{-7}	1.5×10^{-8}	3×10^{-9}	1.7×10^{-7}	2×10^{-6}	2.5×10^{-5}
Mean values...	3.85×10^{-7}	1.6×10^{-8}	3×10^{-9}	2.2×10^{-7}	2.7×10^{-6}	2.5×10^{-5}

correspond exactly to one pure radiation of wave length, 0.55 μ for instance, but to the beam comprised between 0.537 μ and 0.563 μ, the slit covering a range of 0.026 μ.

White Light.—The same technique was applied to the *minimum visibile* for white light (Nernst lamp), and gave 3.8×10^{-11} ergs, for continuous impression by total radiation. This figure agrees well with that of Grijns and Noyons for the Hefner lamp, 4.4×10^{-11}. It is better to compare figures related to total radiation, because these figures do not involve the more or less arbitrary choice of limits of integration and the knowledge of the ratio $\dfrac{L}{R}$ for the considered source.
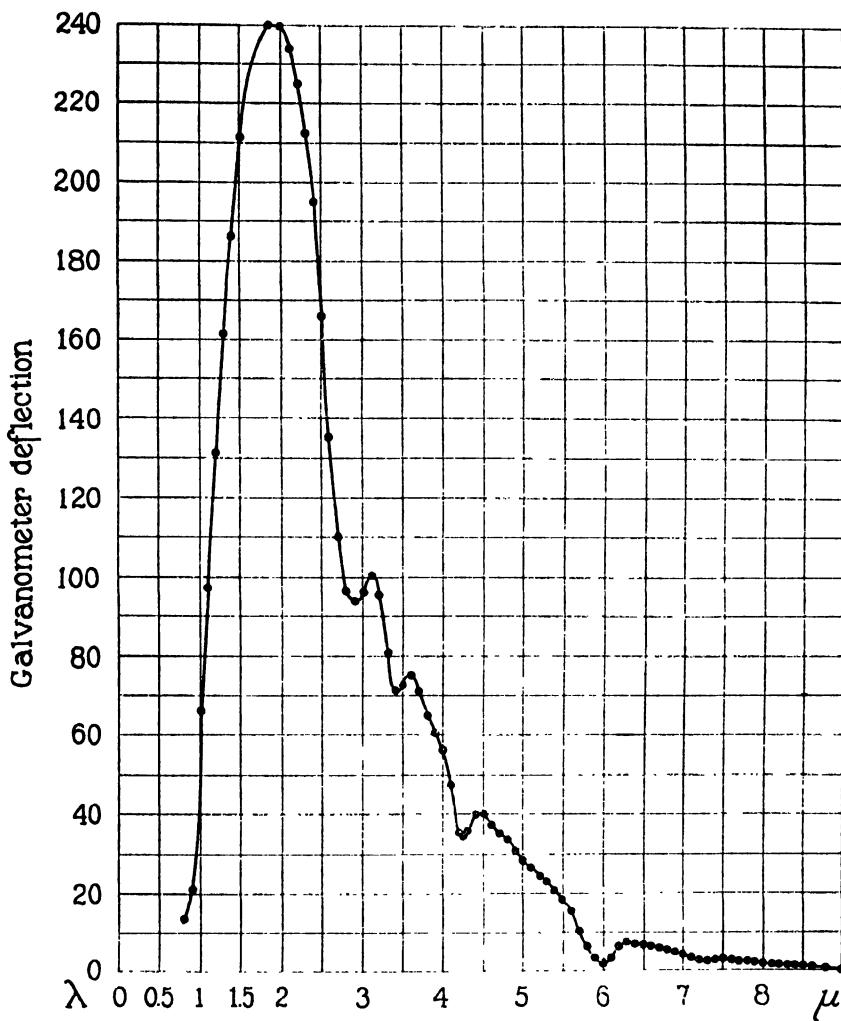
FIG. 6. Energy distribution curve of the Nernst lamp used (uncorrected), 91.8 watts: slits: 0.025 mm.

Criticisms of the Results.—As has been pointed out before by Langley, the errors involved in the determination of the threshold of sensitivity (*minimum visibile*) may be perhaps 100 per cent, or even more. For this reason, the absorption by the various eye

media, for the total depth of the eye, which amounts only to about 1 per cent for 0.7 μ and less than 0.1 per cent below 0.65 μ, are entirely negligible. The eye, for such small amounts of energy as those corresponding to the *minimum visibile*, does not perceive a continuous increase in the brightness of the spot, when its intensity is increased progressively, but seems to react by steps. High authorities, such as Joly, and Henri, disagree entirely as to the explanation of vision on the basis of the quantum theory.

Accuracy of the Method.—In the method used, the following causes of error could be corrected:

Errors Due to the Spectrometer.

1. Selective reflection by the three gilded mirrors.
2. Selective absorption by the rock salt prism.
3. Contraction of the spectrum (non-normal spectrum).

Errors Due to the Use of a Nernst Filament.

1. Uneven distribution of spherical energy.
2. Disturbing effect of volt- and ammeter in the circuit of the glower.
3. Radiant output of glower, (losses by conduction).

The following errors are also involved, and were not corrected:

Errors Due to the Integration Method.

1. Determination of limits of integration, (arbitrary).
2. Material errors due to the mechanical integration of surfaces.

We can probably admit that they do not amount to more than 10 per cent.

Errors Due to the Assumed Quantity of Energy Radiated.

1. Errors due to the fact that the image of the glower was not formed on the collimator slit.
2. Errors due to the emission of radiations from other parts of the instrument.
3. Errors due to the measurement of the total radiation by means of a compensating current.

As our figures are in good accord with those of the best authors, within less than 10 per cent, we may assume that this is the upper limit of error. This gives a total of 20 per cent possible error, which is beyond the possibility of detection by the eye in the *minimum visibile*, as stated before. As some of the individual data differ by more than 100 per cent, the data can only be considered as reliable in the conditions of the experiments, within about 120 to 150 per cent. This is about the order of magnitude of the differences between the experimental data given by Langley.

Quantum Theory.—We can roughly express the *minimum visibile* in function of quanta of energy. For the mean radiation 0.55 μ, the period of the atom is 5.76×10^{14} per second. The minimum of energy perceived is approximately equal to 1.9×10^{-12} ergs per second, (taking 3.8×10^{-11} as the value of the minimum for total radiation, and roughly 5 per cent as belonging to the visible spectrum). Hence,

$$\frac{1.9 \times 10^{-12}}{5.75 \times 10^{4}} = 3.3 \times 10^{-27}$$

As Planck's universal constant $h = 6.5 \times 10^{-27}$, the figure found is satisfactory as far as the order of magnitude is concerned, but it would mean that only one-half quantum per second would be sufficient to cause the luminous sensation; as we have dealt with an area of $\frac{1}{100}$ of a square millimeter on the retina, it would indicate that the destruction of one molecule every 2 seconds on such an area would be sufficient to produce an impression of light.

CONCLUSION.

A method was devised for measuring the *minimum visibile* in different parts of the spectrum, as done by Langley in 1888.

The results are generally in good agreement with those given by this author, although not as close on both sides of the wave length 0.55 μ; this may be due partly to the use of a rock salt prism, to the fact that the minimum was determined by looking at a beam of diffused transmitted, instead of diffused reflected light, and also to the fact that Langley experimented with the sun, through the earth's atmosphere, and had to take into account the thickness of the atmos-

phere interposed and the brightness of the sky. Although his experiments were made with great care, the differences from one day to another are important. However, when he expresses the energy in absolute units, he always refers to the same mean amount of energy radiated by the sun on 1 sq. cm. This amount is certainly not constant, if one judges from the differences observed in two measurements of sensitivity of the eye of the same individual at different dates. On the contrary, for a given wave length, our measurements always agreed closely, as our source of radiation was very nearly constant, owing to the absence of a varying amount of water vapor interposed. This may in some way account for the discrepancies observed.

I wish to express my thanks to Dr. Harry Clark of The Rockefeller Institute for the valuable help he was kind enough to give me in solving certain difficulties which were encountered during this work.

BIBLIOGRAPHY.

1. König, A., cited by Henri, V. and des Bancels, J. L., *J. Physiol. et Path. Gén.*, 1911, xiii, 841.
2. Langley, S. P., *Phil. Mag.*, 1889, xxvii, 1.
3. Grijns, G., and Noyons, A. K., *Arch. Physiol.*, 1905, 25.
4. Zwaardemaker, H., *Ergebn. Physiol.*, 1905, iv, 432.
5. Kries, V., *Z. Sinnesphysiol.*, 1907, xli, 373.
6. Broca, A., *Rec. de Constantes Phys.*, Soc. de Phys., Paris, 1913, 737.
7. Henri, V. and des Bancels, J. L., *J. Physiol. et Path. Gén.*, 1911, xiii, 841.
8. Joly, J., *Phil. Mag.*, 1921, xli, 289.
9. Henri, V., and Wurmser, R., *Compt. Rend. Acad.*, 1913, clvi, 1014.
10. Gage, H. P., *Phys. Rev.*, 1911, xxxiii, 111.
11. König and Brodhun, *Sitzungsber. K. Akad. Wissensch.* 1888, ii, 917; *U. S. Bur. Standards Bull.*, v, 286.
12. Lux, L. W., *Rec. des Constants Phys.*, Paris, 1913, 509.
13. Hartman, L. W., *Phys. Rev.*, 1903, xvii, 65.
14. Ingersoll, L. R., *Phys. Rev.*, 1903, xvii, 371.
15. Nichols, E. L., and Coblentz, W. W., *Phys. Rev.*, 1903, xvii, 267.
16. Ångström, K., *Phys. Rev.*, 1903, xvii, 302.
17. Stewart and Hoxie, cited by Nichols and Coblentz, *Phys. Rev.*, 1903, xvii, 267.
18. Paschen, F., *Ann. Physik.*, 1908, xxvi, 130.
19. Hilger, A. Notes with Regard to the Use of Infra-Red Spectrometer, London. Issued with instrument.
20. Drude, P., Lehrbuch der Optik, 447.

FURTHER OBSERVATIONS ON THE PRODUCTION OF PARTHENOGENETIC FROGS.

By JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, January 19, 1921.)

The writer has repeatedly published[1] short reports on the production of frogs from unfertilized eggs by Bataillon's method of puncturing the membrane of the egg with a fine needle. The writer has succeeded in raising over twenty of these parthenogenetic frogs to an advanced and some to an adult stage. Two such specimens (leopard frogs) are represented in Fig. 1, together with a scale giving their size. They were at the time of death 13 and 14 months old respectively, and the death of these, as of the other specimens, was due to intestinal infection. The parthenogenetic frogs were apparently normal in every respect.

The second point of interest is the fact that both sexes occur among the parthenogenetic frogs. Three females were obtained among over twenty males, yet the preponderance of males may have been simply an accident. Fig. 2 gives a microscopic photograph of ovaries and kidneys of one parthenogenetic female, and Fig. 3 a microphotograph of a section through the ovary. The fact that both sexes occur suggests that in the frog the female may be heterozygous for sex.

It was ascertained that the male parthenogenetic frogs and tadpoles possess a diploid and not a haploid number of chromosomes. The writer had the good fortune of obtaining the expert advice of Professor Richard Goldschmidt,[2] and later of Doctor Parmenter,[3] on this problem. Both authors found unquestionably a diploid number of chromosomes in the males. Parmenter was able to count definitely twenty-

[1] Loeb, J., and Bancroft, F. W., *J. Exp. Zool.*, 1913, xiv, 275; 1913, xv, 379. Loeb, J., *Proc. Nat. Acad. Sc.*, 1916, ii, 313; 1918, iv, 60; The organism as a whole, from a physicochemical viewpoint, New York, 1916.

[2] Goldschmidt, R., *Arch. Zellforsch.*, 1920, xv, 283.

[3] Parmenter, C. L., *J. Gen. Physiol.*, 1919–20, ii, 205.

FIG. 1. Two adult male parthenogenetic leopard frogs, natural size (preserved in formaldehyde). The one to the left lived from Feb. 27, 1917, to Mar. 24, 1918; the one to the right from Mar. 16, 1916, till May 22, 1917.

six chromosomes in a number of specimens.   The question arises how
to account for the fact that the number of chromosomes is diploid.
The first thought might be that the eggs had been accidentally fer-
tilized, but this is excluded by the mode of procedure.   As is well
known, the eggs of the frog are fertilized outside the body of the

FIG. 2. Ovaries and kidneys of a parthenogenetic female frog.

female, and the females used for our experiments had not yet com-
menced to lay their eggs.   The females after having been killed were
submersed in 95 per cent alcohol and left there for several minutes
to kill any sperm that might have stuck to the outside skin.   The
skin was cut open and the eggs were removed from the oviduct with

sterilized instruments. The hands of the experimenter were also sterilized. About 50 to 100 eggs were put on each glass slide and the eggs of every second or third slide were not punctured, serving as controls. In no case did a single control egg show any development,
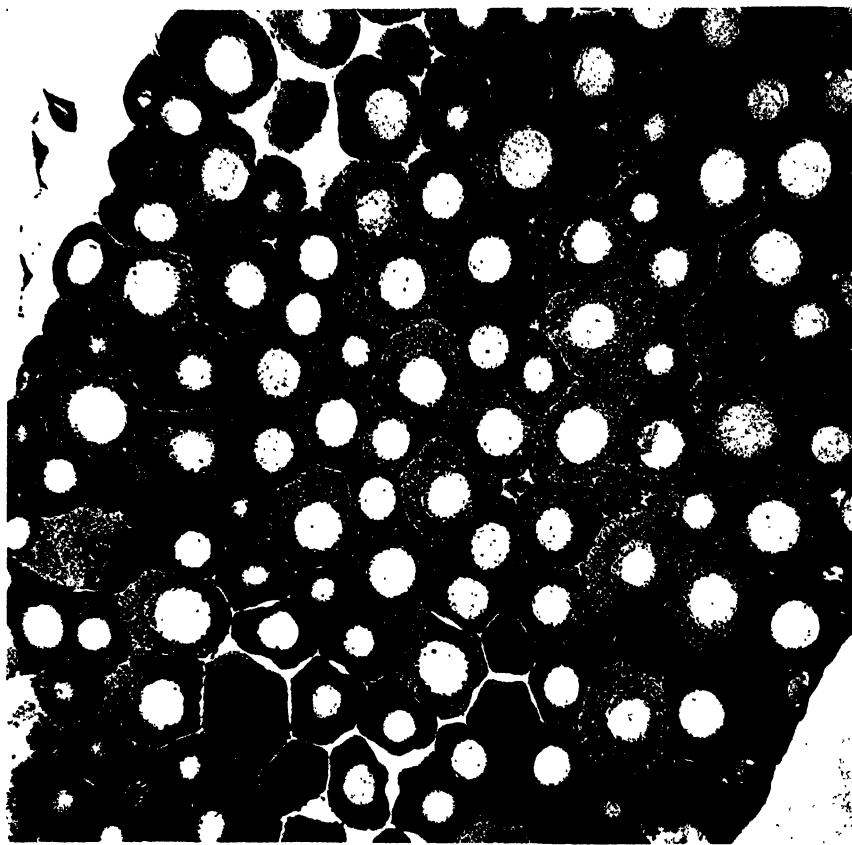
FIG. 3. Section through the ovary shown in Fig. 2.

only punctured eggs developed. This excludes the idea that the eggs were fertilized.

There remain other possibilities to account for the fact that the chromosome number is diploid. As Parmenter states[3]

"The diploid number, as well as the similarity in form of the tetrads of the parthenogenetic and normal animals, may have been brought about by the retention of the second polar body, or by a premature division of the chromosomes without the division of the cell body just before the first cleavage."

Brachet[4] had previously counted the number of chromosomes in a parthenogenetic tadpole, 18 days old, and found a diploid number, but, of course, it was out of the question to ascertain the sex of the tadpole.

In my last publication on the subject I mentioned the possibility that the parthenogenetic females might have the haploid number of chromosomes. This question can only be decided by an actual count of the chromosomes in female parthenogenetic frogs. It is perhaps of interest in this connection that Hovasse[5] in an investigation of the number of chromosomes of young parthenogenetic tadpoles of frogs reports to have found both the diploid number and the haploid number. Since it is not possible to determine the sex of early tadpoles the observations of Hovasse do not answer the question whether or not the female parthenogenetic frog possesses a haploid number of chromosomes. There is, however, no doubt left that some if not all of the parthenogenetic male frogs possess a diploid number of chromosomes.

We usually received in one shipment a large number of female frogs either from South Carolina or from Chicago as soon as the spawning season began. It took about half a day to puncture the eggs of one female frog and as a consequence some time elapsed before the eggs of every female in the lot were punctured. It was found that tadpoles of good vitality were obtained only from the eggs of the first and second frogs used for the experiment. The eggs of the frogs which were punctured later, after the frogs had been in the laboratory for 2 days or more, either gave no tadpoles at all or if tadpoles were produced they died in less than 3 weeks, while the tadpoles from the eggs punctured immediately after the arrival of the frogs went on developing although the conditions under which the eggs were kept were the same in all cases (see Table I).

[4] Brachet, A., *Arch. Biol.*, 1911, xxvi, 362.

[5] Hovasse, R., *Compt. rend. Acad. Sc.*, 1920, clxx, 1211.

TABLE I.

Date of experiment.	Female No.	Approximate No. of eggs punctured.	No. of tadpoles hatching.	No. of tadpoles that died within 3 weeks.
1919				
Mar. 25	I	8,500	92	21
" 26	II	5,000	43	22
" 28	III	3,000	9	9
" 29	IV	3,000	2	2
" 30	V	100	0	0
" 30	VI	400	0	0
" 30	VII	200	0	0
" 31	VIII	1,500	13	13
" 31	IX	150	0	0
" 31	X	1,300	1	1
" 31	XI	No eggs used.		
Apr. 1	XII	2,800	13	13
" 1	XIII	1,700	0	0
" 1	XIV	1,400	0	0
" 2	XV	1,200	16	14
" 2	XVI	200	0	0
" 2	XVII	1,100	1	1
" 3	XVIII	1,800	16	16
" 3	XIX	2,000	16	12
" 4	XX	1,500	0	0
" 4	XXI	None.		
" 4	XXII	1,700	0	0
" 5	XXIII	2,000	0	0

The results of a series of experiments made on the eggs of a second lot of frogs received later confirmed these results (Table II).

TABLE II.

Date of experiment.	Female No.	Approximate No. of eggs punctured.	No. of tadpoles hatched.	No. of tadpoles that died within 3 weeks.
1919				
Apr. 8	XXIV	2,000	6	5
" 8	XXV	4,000	14	14
" 9	XXVI	3,000	2	2
" 9	XXVII	3,500	0	0
" 10	XXVIII	2,000	0	0

It is, therefore, obvious that only those eggs were capable of developing which were punctured on the 1st or 2nd day while later they lost their power of developing or lost their vitality. It may be

possible that the eggs must be punctured at a certain stage, *e.g.* at the period after the first polar body is given off and before the second polar body is extruded in order to develop or in order to develop normally, but this can only be decided by further experiments.

In these experiments the eggs were not covered with blood since it was found in previous experiments that this did not improve the yield of parthenogenetic tadpoles.

Many, sometimes the majority, of the parthenogenetic tadpoles did not metamorphose even within a year although they grew normally. It was suspected that this was due to an imperfect development of the thyroid gland or to lack of iodine in the food. In one tadpole, over a year old, the attempt was made to bring about metamorphosis by the feeding of thyroid gland from cattle. Three feedings were sufficient to cause metamorphosis within 2 weeks.

CHEMICAL AND PHYSICAL BEHAVIOR OF CASEIN SOLUTIONS.

BY JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

1. L. L. Van Slyke and J. C. Baker described in 1918[1] a method for preparing "pure casein" from skimmed milk, which consisted in "the gradual addition of acid and its immediate distribution through the mass of milk without causing coagulation of casein at the point where the acid first comes into contact with a portion of the milk. This result can be accomplished by introducing the acid below the surface of the milk with simultaneous high-speed mechanical stirring. . . . After standing under gentle stirring for 3 hours with acidity just below the point of casein coagulation, addition of acid is continued slowly, accompanied as before by rapid stirring in order to obtain the particles of casein coagulum in the finest possible state of division." The coagulated casein is then centrifuged and after repeated washings is found free from Ca and P. As Van Slyke and Baker point out, the pH of this casein coagulum is about 4.5 to 4.6; *i.e.*, it is slightly below the isoelectric point. The essential feature of Van Slyke and Baker's method, therefore, consists in slowly bringing the milk or casein solution approximately to the pH of the isoelectric point of casein. The writer has shown that gelatin gives off all ionogenic impurities at the isoelectric point[2] and Van Slyke and Baker's experiments show that the same method works also with casein. The casein prepared after Van Slyke and Baker's method is also free from albumin since this latter protein is soluble at pH 4.5 or 4.7, and is hence removed from the insoluble isoelectric casein by washing.

[1] Van Slyke, L. L., and Baker, J. C., *J. Biol. Chem.*, 1918, xxxv, 127.

[2] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 237.

In our experiments we used casein prepared after Van Slyke and Baker's method from skimmed milk and in addition from a commercial "pure casein." Both preparations gave practically the same result. In order to remove traces of fat from the casein the latter was washed in acetone.

2. In previous publications the writer had shown that weak dibasic and tribasic acids combine in molecular proportions with crystalline egg albumin, prepared after Sörensen and with gelatin.[3] It can be shown that the same is true for casein. 1 gm. of isoelectric casein, prepared after Van Slyke and Baker, was put into 100 cc. of watery solution containing 1, 2, 3, etc. cc. of 0.1 N HCl or 0.1 N H_3PO_4. The pH of the casein solution was ascertained potentiometrically and the number of cc. of 0.1 N acid required to bring the 1 per cent casein solution to the same pH was plotted as ordinates over the final pH of the casein solution as abscissæ. The casein chloride or casein phosphate is not completely soluble in a 1 per cent solution at room temperature until the pH is about 3.0 or a trifle below. When too much acid is added, i.e. when the pH is 1.6 or possibly a little above, casein precipitates out again from a 1 per cent solution.

Fig. 1 gives the curves for HCl and H_3PO_4, drawn out within those limits of pH within which the casein salts are soluble in a 1 per cent solution at room temperature. The curves show that about three times as many cc. of 0.1 N H_3PO_4 as of 0.1 N HCl are required to bring 1 gm. of originally isoelectric casein in a 1 per cent solution to the same pH; or in other words, H_3PO_4 combines with casein in molecular proportions, as we should expect if casein phosphate is a true chemical compound.

It was not possible to plot the corresponding curves for casein sulfate and casein oxalate since these salts are too sparingly soluble. This is true also for casein salts with other acids; e.g., triacetic acid.

3. The writer had shown that the influence of different acids on the physical properties of gelatin or crystalline egg albumin depends only upon the valency and not upon the nature of the ion in combination with the protein.[3] Thus the values of osmotic pressure or viscosity of gelatin chloride are identical with those of gelatin phosphate for

[3] Loeb, J., J. Gen. Physiol., 1918–19, i, 559; 1920–21, iii, 85, 247; Science, 1920, lii, 449.

the same pH and the same concentration of originally isoelectric gelatin; and the same is true for crystalline egg albumin. The reason is that in the case of gelatin or albumin phosphate the anion is the
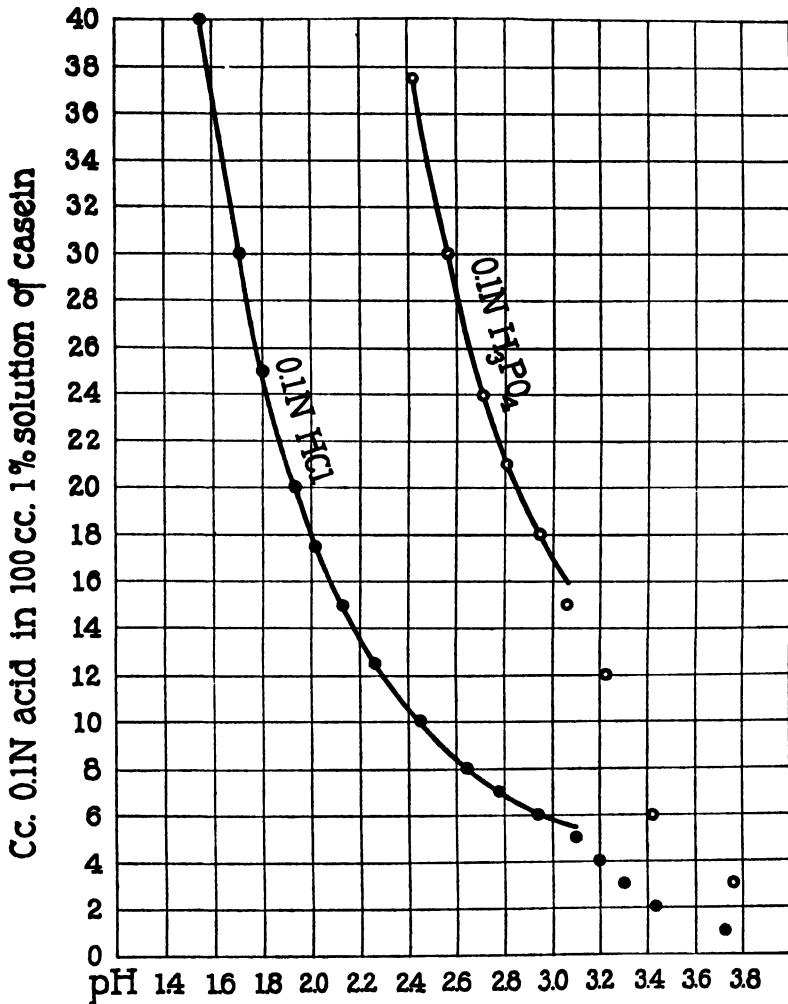
FIG. 1. Ordinates represent the cc. of 0.1 N HCl or H_3PO_4 in 100 cc. of 1 per cent casein solution. The abscissæ are the pH of the solution. Approximately three times as many cc. of 0.1 N H_3PO_4 as of 0.1 N HCl are required to bring 1 gm. of casein to the same pH.

monovalent anion H_2PO_4 and not the trivalent anion PO_4. If the same rule holds for casein, the osmotic pressure and viscosity of casein phosphate should be practically identical with that of casein chloride when plotted over the same pH and when the concentration of (originally isoelectric) casein is the same in both cases (1 gm. in 100 cc. of solution). Fig. 2 shows that the osmotic pressure curves for casein chloride and casein phosphate (in 1 per cent solutions) are
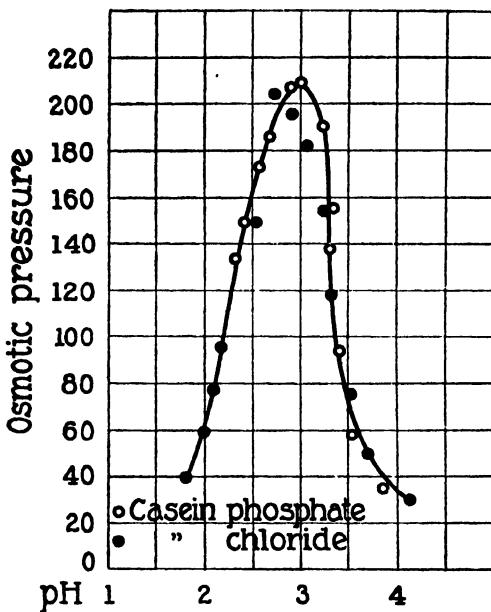
FIG. 2. Osmotic pressure of 1 per cent solutions of casein chloride and casein phosphate as function of pH. The two curves are almost identical.

almost identical. The curve includes also the osmotic pressure at a pH between 4.0 and 3.0 where the two casein salts are not completely soluble in 1 per cent solutions, but since the relative solubilities of casein chloride and casein phosphate are also practically identical, the osmotic pressure curves for the pH where the solubility of the two salts is not complete remain approximately the same.

Fig. 3 gives the viscosity curve for 1 per cent solutions of casein chloride and casein phosphate over pH as abscissæ. The ordinates

are the quotients of the time of outflow of the casein solutions over the time of outflow of distilled water through the same viscometer. The time of outflow for distilled water was 60 seconds. We will, for the sake of brevity, call this ratio specific viscosity. We see that this specific viscosity does not rise above 1 as long as the pH is above
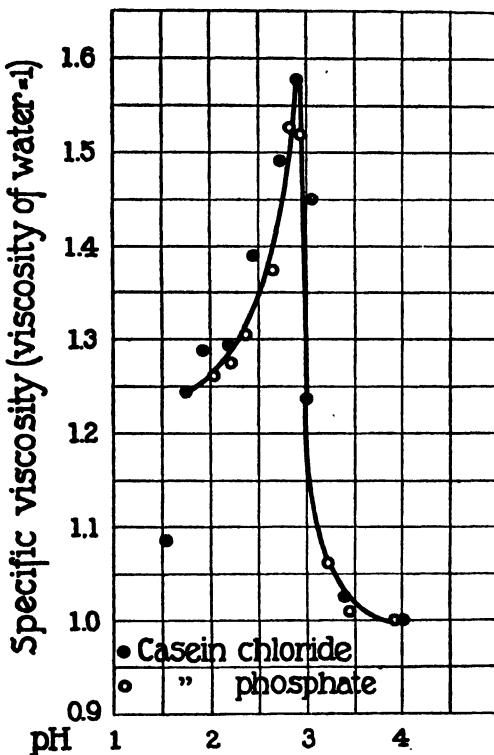
FIG. 3. Viscosity of 1 per cent solutions of casein chloride and casein phosphate. The curves are approximately identical.

3.5. At a pH of 3.0 a sharp rise occurs, because the solubility of the casein increases at this point considerably. With a further fall of pH the viscosity diminishes again. Fig. 3 shows that the viscosity curves for casein chloride and casein phosphate are almost identical, which was to be expected if the rules found for gelatin and crystalline egg albumin are also true for casein.

We had shown that when we add $Ca(OH)_2$ or $Ba(OH)_2$ to isoelectric gelatin or isoelectric crystalline egg albumin the two alkalies combine with the protein in equivalent proportion.[3] Hence the same number
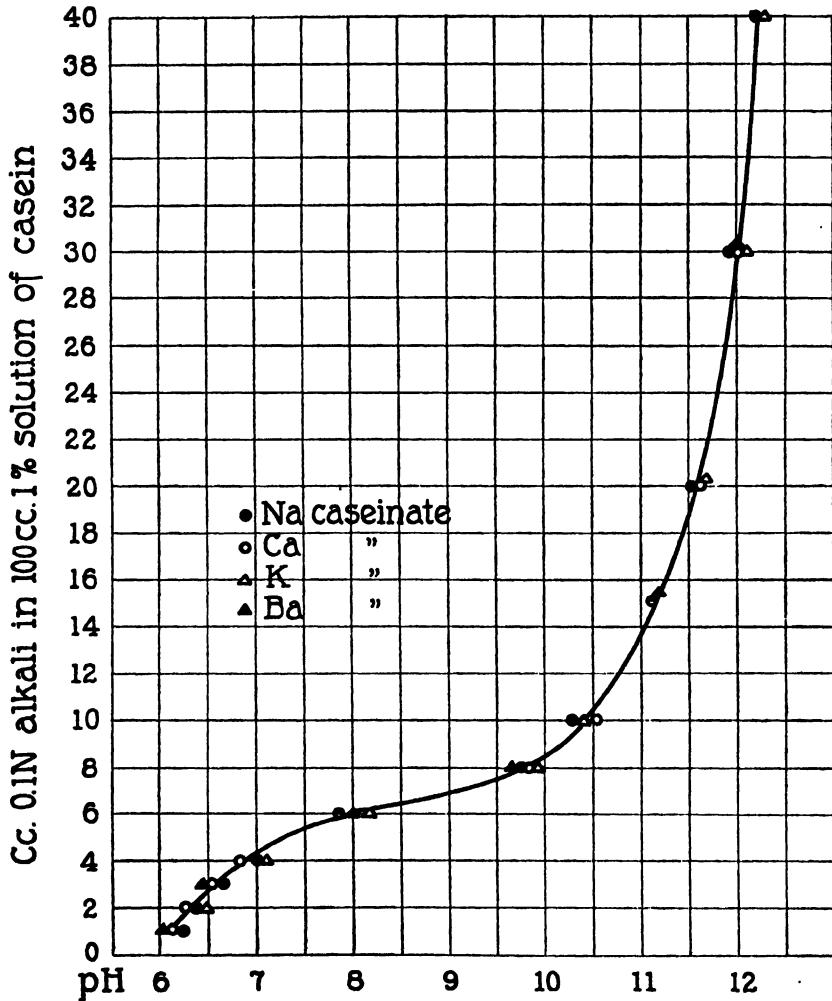
FIG. 4. Ordinates are the cc. of 0.1 N NaOH, KOH, $Ca(OH)_2$, and $Ba(OH)_2$ in 100 cc. of 1 per cent solution of casein. Abscissæ are the pH of the solution. The curves for the four alkalies are identical, proving that Ba and Ca combine with casein in equivalent proportion.

of cc. of 0.1 N Ba(OH)₂ or Ca(OH)₂ was required to bring a 1 per cent
solution of isoelectric gelatin or crystalline egg albumin to a given
pH as was required in the case of NaOH or KOH. It can be shown
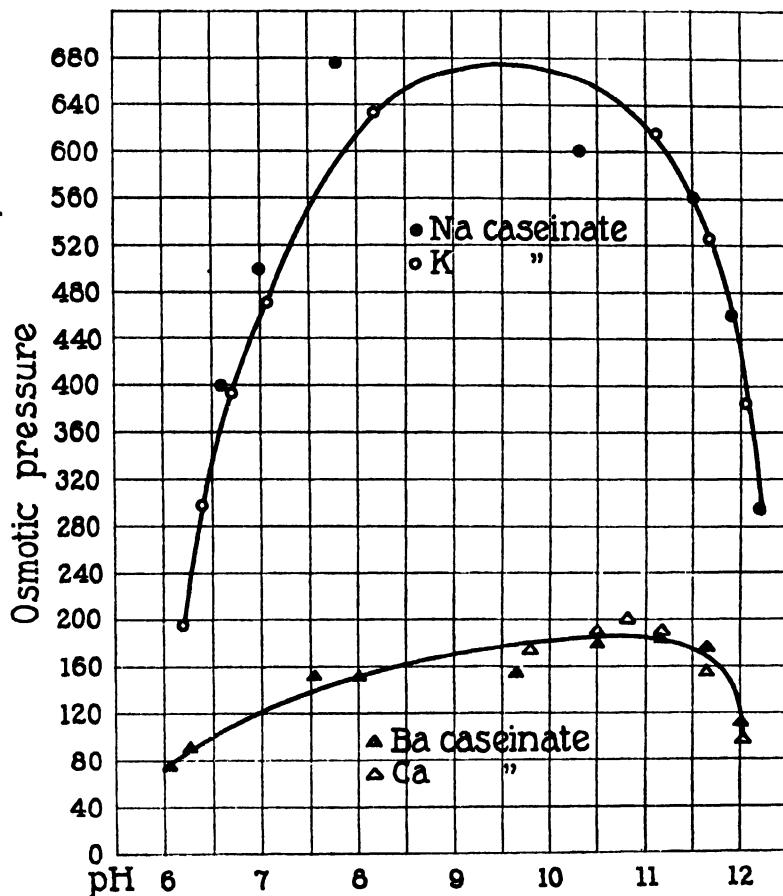
FIG. 5. Osmotic pressure of Na caseinate, K caseinate, Ba caseinate, and Ca
caseinate. The curves for the former two caseinates are identical and consider-
ably higher than those for the latter two caseinates.

that the same is true for casein. In Fig. 4 the abscissæ are the pH,
the ordinates the number of cc. of 0.1 N NaOH, KOH, Ca(OH)₂,
and Ba(OH)₂ that must be contained in 100 cc. of a 1 per cent solution

of isoelectric casein to bring it to the same pH. The curves for the four alkalies are identical (Fig. 4), thus proving that Ca(OH)$_2$ and Ba(OH)$_2$ combine with casein in equivalent proportion. Table I gives the limits of pH between which the four metal caseinates are completely soluble in a 1 per cent solution at room temperature.

TABLE I.

1	per	cent	Na	caseinate	completely	soluble	between	pH	7.02	and	>	12.20
1	"	"	K	"	"	"	"	"	7.09	"	>	12.28
1	"	"	Ca	"	"	"	"	"	10.53	"	>	12.00
1	"	"	Ba	"	"	"	"	"	10.50	"	>	12.26
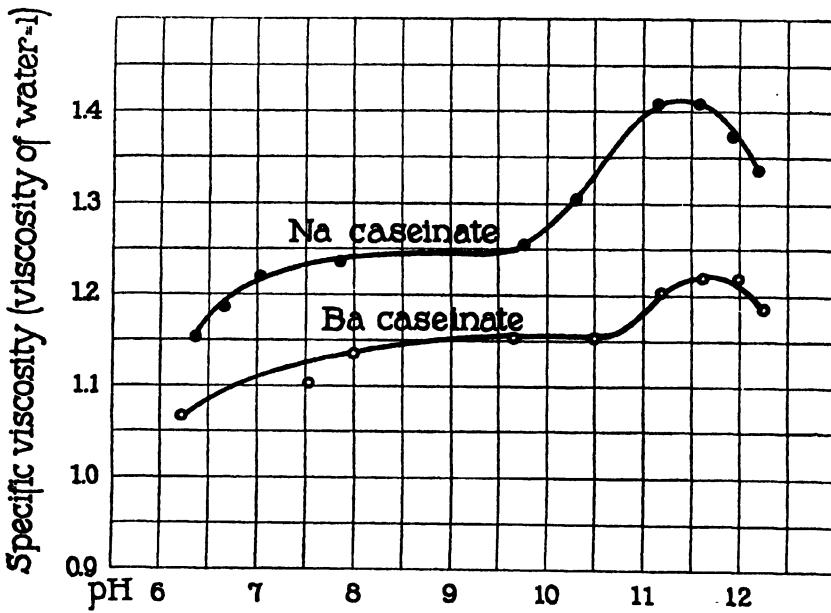
FIG. 6. Viscosity curves for Na caseinate and Ba caseinate.

On account of the incomplete solubility of Ba caseinate and Ca caseinate at a pH between 4.7 and 10.5 it is not possible to draw any conclusion from the relative osmotic pressure or relative viscosity of metal caseinates with monovalent and divalent cation between a pH of 4.7 and 10.5. This should be kept in mind in judging the curves in Figs. 5 and 6.

When we plot the osmotic pressure of these solutions as ordinates over the pH as abscissæ (Fig. 5), we notice that the curves for the osmotic pressure of Na and K caseinate are alike; the curves for the casein salts are, however, over three times as high when the cation is monovalent (Na or K) than when the cation is bivalent (Ca or Ba). This is, however, chiefly the result of the fact that Ca and Ba caseinate are incompletely soluble up to a pH of 10.5.

Fig. 6 gives the viscosity curves for Na caseinate and Ba caseinate. The difference in height between the two casein salts is between pH 11 and 12 of a similar order as in the case of Na and Ba gelatinate.

SUMMARY AND CONCLUSION.

The experiments on casein solutions therefore confirm the conclusion at which we arrived from the behavior of gelatin and crystalline egg albumin that the forces determining the combination between proteins and acids or alkalies are the same forces of primary valency which also determine the reaction between acids and alkalies with crystalloids, and that the valency and not the nature of the ion in combination with a protein determines the effect on the physical properties of the protein.

THE COLLOIDAL BEHAVIOR OF PROTEINS.

BY JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

I.

Colloids show a number of peculiarities which at first appearance seem to be lacking in crystalloids, and these properties are generally accounted for by differences in the degree of dispersion. We have repeatedly discussed these peculiarities in our analysis of the chemical behavior of proteins, *e.g.* the depressing effect of neutral salts on the osmotic pressure, swelling, and viscosity of certain proteins; the peculiar influence of the hydrogen ion concentration of the solution on these properties; and finally the peculiar influence of the valency of the ion with which the protein is in combination and the apparent lack of influence of the other chemical properties of the ion except valency and sign of charge.[1] The dispersion theory accounts for these difficulties by the assumption of differences in the degree of aggregation of the protein particles. If, for example, the addition of some salt to a protein solution of a definite pH lowers its osmotic pressure, the assumption is made that the salt diminishes the degree of dispersion of the colloidal particles in solution. It is not possible to submit the dispersion theory to a quantitative test since we cannot measure the degree of dispersion of a protein.

A second theory to account for the influence of salts and hydrogen ion concentration is Pauli's ionization theory which ascribes the osmotic pressure, the swelling, and viscosity chiefly to the hydratation of ionized protein, while the non-ionized protein molecule is assumed not to be hydrated. The idea of such a hydratation of protein ions has become doubtful in view of recent experimental and theoretical investigations by Lorenz[2] and by Born,[3] and, moreover, the writer

[1] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 85, 247, 391; *Science*, 1920, lii, 449.

[2] Lorenz, R., *Z. Elektrochem.*, 1920, xxvi, 424.

[3] Born, M., *Z. Elektrochem.*, 1920, xxvi, 401.

has been able to show that the conductivity measurements of protein solutions contradict Pauli's ionization theory.

The solution of the problem seems to lie in a field altogether foreign to the speculations current in colloid chemistry, namely in the Donnan equilibrium, which exists when a membrane separates two solutions, one of an electrolyte for the ions of which the membrane is permeable, and one of an electrolyte for one ion of which the membrane is not permeable.[4] It is immaterial whether the latter ion is a colloid or a crystalloid; it is only necessary that it cannot diffuse through the membrane. When a collodion membrane separates a gelatin or albumin chloride solution of pH 3.3 from an aqueous solution of HCl of originally the same pH (but without gelatin), the pH is no longer the same on both sides of the membrane at the time of equilibrium but is lower on the outside than in the gelatin solution. The Donnan equilibrium demands in this case that acid be given off from the gelatin chloride solution to the outside aqueous solution (containing no gelatin). The writer has found that, *e.g.* a 1 per cent gelatin chloride solution of pH 3.5 is in equilibrium with an aqueous HCl solution of about pH 3.0.[5]

<center>II.</center>

Gelatin chloride solutions containing 1 gm. of originally isoelectric gelatin in 100 cc. solution and having a pH of 3.5 were made up in H_2O and in different concentrations of $NaNO_3$ varying from $M/4,096$ to $M/32$ $NaNO_3$, all of pH of 3.5. These solutions were put into collodion bags connected with a manometer to measure the final osmotic pressure of the solution. The collodion bags were put into HCl solution of pH 3.0 made up in water and different concentrations of $NaNO_3$, the pH of the $NaNO_3$ solutions being also 3.0. These outside solutions contained no gelatin. The collodion bags were put into these aqueous solutions free from gelatin in such a way that the concentration of the $NaNO_3$ solution inside the collodion bag was always the

[4] Donnan, F. G., *Z. Elektrochem.*, 1911, xvii, 572. Donnan, F. G., and Harris, A. B., *J. Chem. Soc.*, 1911, xcix, 1554. Donnan, F. G., and Garner, W. E., *J. Chem. Soc.*, 1919, cxv, 1313.

[5] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 247.

same as outside. The osmotic pressure of the 1 per cent gelatin chloride solution, which was after 18 hours 435 mm. when no salt was present, was only 63 mm. when the inside and outside solutions were made up in M/32 NaNO₃.

Table I gives the influence of the concentration of NaNO₃ on the osmotic pressure. It is obvious that the osmotic pressure diminishes with the concentration of the salt. This phenomenon had already been described by Lillie[6] and by the writer.[7] Donnan has shown that this depressing effect of a salt on the osmotic pressure of a colloidal solution is a necessary consequence of his theory of membrane equilibrium, and this conclusion is supported by the following experiments.

TABLE I.

Original inside solution, 1 per cent gelatin chloride of pH 3.5 made up in various concentrations of NaNO₃ of the same pH.

Outside solution, HCl of pH 3.0 made up in the same concentrations of NaNO₃ of pH 3.0 as the inside solution.

	Concentration of NaNO₃.								
	0	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32
Osmotic pressure in mm. of H₂O..	435	405	371	335	280	215	134	85	63
P.D. inside solution in millivolts..........	+31	+26	+24	+22	+16	+12	+7	+4	0

The writer undertook measurements of the potential difference between the gelatin chloride solutions inside the collodion bag and the aqueous solutions outside the collodion bag with the aid of a Compton electrometer. It was found that the gelatin chloride solution was always positively charged while the outside aqueous solution was negatively charged, as was to be expected. The second important fact is that the P.D. diminishes with the increase in concentration of the neutral salt added and (if the necessary corrections are made) in approximately the same ratio as the osmotic pressure diminishes (lower row in Table I).

[6] Lillie, R. S., *Am. J. Physiol.*, 1907–08, xx, 127.

[7] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 391.

III.

What is the origin of these potential differences? Beutner[8] has shown that the potential differences at the boundary of water and water-immiscible substances obey Nernst's well-known logarithmic formula

$$E = \frac{RT}{nF} \ln \frac{C_1}{C_2}$$

at room temperature and for $n = 1$

$$0.058 \log \frac{C_1}{C_2}$$

Loeb and Beutner have found the same formula to hold for the potential differences at the boundary of water and the skin of an apple, a tomato, or the leaf of a rubber plant.

It can be shown that the potential differences mentioned in Table I follow Nernst's formula, if we assume that only the hydrogen ion concentration need be considered for the potential difference. If C_1 is the concentration of free HCl in the gelatin solution, and C_2 the concentration of HCl in the outside aqueous solution (without gelatin) the value $\log \frac{C_1}{C_2}$ becomes equal to (pH inside − pH outside).

We measured the pH of the gelatin chloride solution (inside solution) and of the outside HCl solution (without gelatin) after the osmotic and the Donnan equilibria were established. The surprising result was noticed that the difference of pH inside the gelatin solution minus the pH of the outside solution (without gelatin) becomes the smaller the greater the concentration of the $NaNO_3$, as shown in Table II.

We can calculate from this difference of pH inside minus pH outside the P.D. between inside and outside solution in millivolts by multiplying the differences by 58 or 59 (correcting for temperature of 24°C.). If the Nernst formula holds the values for P.D. thus calculated should be identical with the observed values for P.D. Table III shows that this is true to a remarkable degree.

[8] Beutner, R., Die Entstehung elektrischer Ströme in lebenden Geweben, Stuttgart, 1920.

TABLE II.

pH Inside Minus pH Outside after 18 Hours.

Original inside solution, 1 per cent originally isoelectric gelatin dissolved in various concentrations of $NaNO_3$ made up with HCl to pH 3.5.

Outside solution, same concentrations of $NaNO_3$ all made up with HCl to pH 3.0.

	Concentration of $NaNO_3$.								
	0	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32
pH of inside solution.......	3.58	3.56	3.51	3.46	3.41	3.36	3.32	3.29	3.25
" " outside "	3.05	3.08	3.10	3.11	3.14	3.17	3.20	3.22	3.24
Difference pH inside minus pH outside.............	0.53	0.48	0.41	0.35	0.27	0.19	0.12	0.07	0.01

TABLE III.

Potential Difference between Gelatin Solution and Outside Solution.

Concentration of $NaNO_3$.	Calculated by Nernst's formula from pH.	Observed.
	millivolts	*millivolts*
0	31.2	. 31
M/4,096	28.3	28
M/2,048	24.0	24
M/1,024	20.7	22
M/512	16.0	16
M/256	11.2	12
M/128	7.0	7
M/64	4.1	4
M/32	0	0

IV.

The greatest puzzle in the physical chemistry of the proteins is the fact that at the isoelectric point of gelatin and crystalline egg albumin the osmotic pressure is a minimum, that it rises when acid is added, at first with the increase of acid added, reaching a maximum when the pH is about 3.5, and that on further addition of acid a rapid fall of osmotic pressure occurs.

It was also reported in the preceding papers that the osmotic pressure of gelatin chloride and gelatin phosphate solutions of the same pH and the same concentration of originally isoelectric gelatin was about the same, that the osmotic pressure of gelatin oxalate was slightly

lower, while that of gelatin sulfate was only half as high or not quite half as high as that of gelatin chloride.

Measurements of the potential differences between the gelatin solution and the outside solution revealed the fact that the curves presenting the P.D. as a function of the hydrogen ion concentration resemble the curves for osmotic pressure. The P.D. curves have a minimum at the isoelectric point, rise steeply with increasing hydrogen ion concentration until a pH of 3.9 is reached, then drop equally steeply again when the pH falls further. Moreover, the maximum of the P.D. curve for gelatin sulfate is about one-half of that of the maximum of the P.D. curve for gelatin chloride and the P.D. curve for gelatin chloride is about equal to the P.D. curve for gelatin phosphate, while that of gelatin oxalate is only slightly lower than that of gelatin chloride.

The next question was whether or not the Nernst formula can account for these differences. The pH of the gelatin solutions and of the outside solutions were measured at the point of equilibrium and it was found that the difference of pH inside minus pH outside multiplied by 58 gave approximately the number of millivolts actually measured. The agreement between the calculated P.D. and the observed P.D. was not as perfect as in the case of the salt effect (Table III) but sufficient to leave no doubt that Nernst's theory accounts for these P.D.

<div align="center">V.</div>

Procter[9] has applied the Donnan equilibrium to the theory of swelling of gelatin, reaching the conclusion that swelling is an osmotic phenomenon and that the amount of swelling of a gelatin chloride solution is determined by the concentration of the free ions inside the gel minus the concentration of the free ions in the outside solution. He did not measure the pH. By filling this gap the writer was able to satisfy himself that the depressing influence of salts upon the swelling of gelatin is due to a diminution of the difference of pH inside

[9] Procter, H. R., *J. Chem. Soc.*, 1914, cv, 313. Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307.

and outside the gel, and that the curves expressing the influence of neutral salts on the value of pH inside minus pH outside the gel, and on the swelling run approximately parallel.

1. It is well known that neutral salts depress the osmotic pressure swelling, and viscosity of protein-acid salts. Measurements of the P.D. between gelatin chloride solutions contained in a collodion bag and an outside aqueous solution show that the salt depresses the P.D. in the same proportion as it depresses the osmotic pressure of the gelatin chloride solution.

2. Measurements of the hydrogen ion concentration inside the gelatin chloride solution and in the outside aqueous solution show that the. difference in pH of the two solutions allows us to calculate the P.D quantitatively on the basis of the Nernst formula $E = \dfrac{RT}{nF} \ln \dfrac{C_1}{C_2}$ if we assume that the P.D. is due to a difference in the hydrogen ion concentration on the two sides of the membrane.

3. This difference in pH inside minus pH outside solution seems to be the consequence of the Donnan membrane equilibrium, which only supposes that one of the ions in solution cannot diffuse through the membrane. It is immaterial for this equilibrium whether the non-diffusible ion is a crystalloid or a colloid.

4. When acid is added to isoelectric gelatin the osmotic pressure rises at first with increasing hydrogen ion concentration, reaches a maximum at pH 3.5, and then falls again with further fall of the pH. It is shown that the P.D. of the gelatin chloride solution shows the same variation with the pH (except that it reaches its maximum at pH of about 3.9) and that the P.D. can be calculated from the difference of pH inside minus pH outside on the basis of Nernst's formula.

5. It was found in preceding papers that the osmotic pressure of gelatin sulfate solutions is only about one-half of that of gelatin chloride or gelatin phosphate solutions of the same pH and the same concentration of originally isoelectric gelatin; and that the osmotic pressure of gelatin oxalate solutions is almost but not quite the same as that of the gelatin chloride solutions of the same pH and concentra-

tion of originally isoelectric gelatin. It was found that the curves for the values for P.D. of these four gelatin salts are parallel to the curves of their osmotic pressure and that the values for pH inside minus pH outside multiplied by 58 give approximately the millivolts of these P.D.

In this preliminary note only the influence of the concentration of the hydrogen ions on the P.D. has been taken into consideration. In the fuller paper, which is to follow, the possible influence of the concentration of the anions on this quantity will have to be discussed.

The writer wishes to express his indebtedness to his technical assistants, Mr. M. Kunitz and Mr. N. Wuest, who have carried out the measurements in these experiments.

THE MECHANISM OF AN ENZYME REACTION AS EXEMPLIFIED BY PEPSIN DIGESTION.[1]

BY JOHN H. NORTHROP.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

One of the most striking peculiarities of living matter is the fact that nearly all the reactions which take place in the organism are due to enzymes. The mechanism of enzyme reactions is therefore very closely connected with the mechanism of the living cell. Many enzyme reactions, however, may be studied *in vitro* and are therefore amenable to quantitative study. The present paper is an attempt to show that the peculiarities of a typical enzyme reaction, pepsin digestion, may be explained by the accepted laws of chemical reactions and that the apparent divergencies from these laws are due to the fact that the enzyme as well as the protein with which it reacts exist in solution as equilibrium mixtures, consisting, in the case of the protein of ionized and non-ionized protein, and in the case of the pepsin of free and combined pepsin. The influence of the various factors on the digestion are primarily due to changes in these equilibria.

It is well known that enzyme reactions in general have certain peculiarities which distinguish them from ordinary chemical reactions. These may be briefly stated as follows:

1. The final amount of change caused by the enzyme is independent of the amount of enzyme present.

2. The rate of change may or may not be proportional to the concentration of enzyme present.

3. The rate of change is proportional to the substrate concentration in dilute solution but increases less rapidly than the substrate concentration in solution of higher concentration.

4. The amount of substrate decomposed in the same time interval by varying enzyme concentrations is not always proportional to the

[1] The experimental data on which this paper is based may be found in *J. Gen. Physiol.*, 1918-19, I., 607; 1919-20, II., 113, 465, 471, 595; 1920-21, III., 211.

concentration of enzyme but is often proportional to the square root of this quantity (Schütz's rule).

5. The reaction proceeds most rapidly at a certain definite hydrogen ion concentration.

It has been found in a study of pepsin digestion that the above peculiarities may be quantitatively accounted for on the basis of the following mechanism.

1. The protein reacts with the acid of the solution to form an ionized protein salt. The amount of this salt formed is determined by the hydrogen ion concentration of the solution according to the well-known laws governing the reaction of an acid and a weak base.

2. The pepsin is present in the solution (a) as free probably negatively charged pepsin, and (b) in combination with the products of hydrolysis of the protein. These two forms are in equilibrium with each other and their relative concentration depends on the amount of products of hydrolysis present in the solution as demanded by the law of mass action.

3. The reaction takes place between the ionized protein and the free pepsin.

EXPERIMENTAL EVIDENCE FOR THE ABOVE STATEMENTS.

Loeb[2] has shown by direct experiment that the protein exists in solution in an equilibrium condition as stated under (1).

Pekelharing and Ringer[3] have shown that purified pepsin in solution is negatively charged. It may be shown by direct experiment that the addition of products of hydrolysis decrease the activity of the enzyme and that the amount of the decrease in the activity can be predicted by the law of mass action.

The validity of the third assumption may best be tested by applying the proposed mechanism to the explanation of the characteristic peculiarities of the reaction outlined under (1 to 5).

1. *Influence of Quantity of Enzyme on the Final Equilibrium.*— Since the free enzyme and the products of hydrolysis are in equilibrium there will always be some active (free) enzyme present no

---

[2] Loeb, J., *J. Gen. Physiol.*, 1918–19, I., 1919–20, II.

[3] Pekelharing, C. A., and Ringer, W. E., *Z. physiol. Chem.*, 1911, LXXV., 282.

matter how high the concentration of products becomes. The reaction will therefore proceed to approximately the same point irrespective of the amount of enzyme present at the beginning of the reaction. It will be seen, however, that the final equilibrium will depend to a slight extent on the amount of enzyme present since some of the products of hydrolysis are combined with the enzyme.

2. *Concentration of Enzyme.*—If the enzyme solution is pure, the rate of hydrolysis, other factors being constant, will be directly proportional to the concentration of enzyme taken. If the enzyme solution contains products of hydrolysis or other substances with which the enzyme is combined then the rate of hydrolysis will increase more slowly than the concentration of enzyme solution, since the amount of free enzyme present becomes relatively smaller the higher the concentration.

3. *Concentration of Protein.*—If the rate of hydrolysis of the protein is proportional to the concentration of *ionized* protein then the rate must increase more slowly than the total protein concentration since the ionization of the protein salt is less in concentrated than in dilute solution.

4. *Schütz's Rule.*—Arrhenius[4] has pointed out that in an equilibrium system such as exists between free pepsin and the products of hydrolysis, the concentration of one of the reacting molecules or ions becomes inversely proportional to the concentration of the second as soon as the second is present in large excess. That is, the amount of free pepsin present, after the first few minutes of the reaction, is inversely proportional to the amount of products formed. It follows from this that the amount of hydrolysis at any time is proportional to the square root of the time elapsed, which is one form of Schütz's rule.

5. *The Influence of the Hydrogen Ion Concentration.*—It is clear that the more acid is added to the protein the more protein salt will be formed until all the protein is present in the form of protein-acid salt. This salt is practically completely ionized in dilute solution as may be shown by direct measurement of the anion concentration

[4] Arrhenius, S., "Quantitative Laws in Biological Chemistry," London, 1915, pp. 36–48.

by means of concentration cells. A further increase in the amount of acid will now serve to decrease the concentration of protein ions by increasing the concentration of the common anion. The concentration of ionized protein will therefore pass through a maximum which should coincide with the maximum for the rate of digestion. If the ordinary theory of chemical kinetics, on the basis of the law of mass action, be applied to the above system, it may be predicted that:

I. The optimum hydrogen ion concentration for the digestion of the protein must coincide with the hydrogen ion concentration at which the concentration of protein ions and therefore the conductivity due to the protein is at a maximum.

II. The limiting pH for the activity of pepsin on the alkaline side must depend on the isoelectric point of the protein, since this is the point at which the protein first begins to react with the acid.

III. The addition of a salt with the same anion as the acid to a solution already containing the optimum amount of acid will have the same depressing effect on the digestion as the addition of the same amount of anion in the form of acid.

IV. The pepsin should combine with the protein only when the latter is ionized, *i.e.*, pepsin should behave the same as the inorganic anions studied by Loeb.

These predictions have been tested quantitatively and found to be fulfilled. It has also been found by direct experiment that neither the influence of the acidity on the destruction of the enzyme, nor the viscosity of the protein solution can account for the influence of the hydrogen ion concentration on the rate of digestion.

It will be seen that from this point of view pepsin digestion is a chemical reaction in which the pepsin as well as the protein takes part. It is therefore not a catalytic reaction at all in the classical sense. The specificity of the reaction is therefore probably governed by the same conditions that determine the specificity of any chemical reaction, since from a quantitative standpoint each chemical reaction is specific. It may be added that a very similar mechanism was proposed by Stieglitz and his collaborators for the hydrolysis of the amido esters by acid.

It is, of course, impossible at present to apply these results directly to the activities of the living organism since conditions there are much more complex. It is probable, however, that much of the apparent complexity is due to the fact that several processes, each simple in itself, occur simultaneously and thus lead to a complicated result. Dernby's[5] experiments render it probable that the phenomenon of autolysis may be explained in this way.

[5] Dernby, K. G., *Biochem. Z.*, 1917. LXXXI., 198.

DONNAN EQUILIBRIUM AND THE PHYSICAL PROPERTIES OF PROTEINS.

I. MEMBRANE POTENTIALS.

BY JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

I. INTRODUCTION.

The different physical properties of proteins, such as osmotic pressure, swelling, and viscosity, vary in a similar way under the influence of changes in the concentration of hydrogen ions or under the influence of neutral salts or under the influence of the valency of the ion in combination with the protein. This fact suggests that the variations of these three physical properties may have a common cause, so that if this cause were known for one of these properties it would, perhaps, be known for all of them. The clues tentatively suggested have thus far either proved wrong or inaccessible to quantitative tests.

In the last paper[1] the writer showed that a measurable potential difference is found when we separate a protein solution from a watery solution (free from protein) by a collodion membrane. When the protein in solution is a protein-acid salt (*e.g.*, gelatin chloride) the protein solution is positive and the water is negative; when the protein exists in the form of a metal proteinate the protein solution is negative and the watery solution positive. The turning point seems to lie at the isoelectric point. Quantitative measurements of these P.D. between gelatin chloride solutions and the outside watery solution with which the gelatin chloride solution was in osmotic equilibrium revealed the fact that these potential differences varied in a similar way as the osmotic pressure, the swelling, or the viscosity

[1] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 557.

when a neutral salt was added or when the hydrogen ion concentration was altered or when the valency of the ion in combination with the protein changed.   This in itself would have meant only the addition of another property varying in the same characteristic way as osmotic pressure, swelling, or viscosity if it had not been for the fact that it was possible to correlate the origin of this new property with Donnan's membrane equilibrium.   Donnan's membrane equilibrium[2] is established when a membrane separates two solutions of electrolytes one of which has one ion for which the membrane is impermeable, while all the other ions can diffuse through the membrane.   It is not necessary that this non-diffusible ion should be a colloid, it may just as well be a crystalloid; all that is required is that it be impossible for this ion to diffuse through the membrane.   The protein ions generally satisfy this condition and a collodion membrane properly prepared is a membrane impermeable to a protein ion.

When equilibrium is established in such a system the distribution of the ions is not the same on both sides of the membrane and from thermodynamic considerations Donnan was able to develop the equations for the relative concentration of the different ions on opposite sides of the membrane at equilibrium.   When a collodion bag filled with a 1 per cent gelatin chloride solution is dipped into a beaker containing a solution of HCl (without gelatin) of the same pH as that of the gelatin solution, the concentration of the hydrochloric acid becomes greater outside than inside the gelatin solution.   The Donnan equilibrium demands that free acid be expelled from the gelatin solution into the outside solution and this actually occurs.[3]

Procter[4] has proposed an osmotic theory of the swelling of gelatin chloride on the assumption that the swelling is a purely osmotic phenomenon.   He deduces from Donnan's theory that at the point

[2] Donnan, F. G., Z. Elektrochem., 1911, xvii, 572.   Donnan, F. G., and Harris, A. B., J. Chem. Soc., 1911, xcix, 1554.   Donnan, F. G., and Garner, W. E., J. Chem. Soc., 1919, cxv, 1313.

[3] This is possible on account of the fact that a gelatin chloride solution always contains free HCl and that there seems to be a chemical equilibrium inside the gelatin chloride solution between the free HCl, the gelatin chloride, and the non-ionogenic gelatin.

[4] Procter, H. R., J. Chem. Soc., 1914, cv, 313.   Procter, H. R., and Wilson, J. A., J. Chem. Soc., 1916, cix, 307.

of equilibrium the relative distribution of HCl inside and outside the solid block of gelatin chloride is determined by the following equation:

$$x^2 = y (y + z)$$

where x is the concentration of H ions (and of Cl ions) outside the gelatin, y the concentration of the H (and Cl) ions of the free HCl inside the gelatin, and z the concentration of the Cl ions in combination with the gelatin ions. Since all quantities are positive, it follows that x must be greater than y; i.e., the concentration of free HCl in the outside solution must be greater than in the gel. The writer has shown that exactly the same happens when we separate a solution of gelatin chloride from pure water by a collodion membrane.

This difference in the concentration of acid on the two sides of the membrane leads to a difference in P.D. at the boundary of the membrane.[5]

This can be proved by the fact that it is possible to calculate the P.D. of the system

| − | outside solution (free from gelatin) concentrated HCl | collodion membrane | inside solution (gelatin chloride) dilute HCl | + |

with a fair degree of accuracy on the basis of Nernst's well known logarithmic formula from the difference of the hydrogen ion concentration on the opposite sides of the membrane. On the basis of Nernst's formula for concentration cells the P.D. is at a temperature of 24°C. $0.059 \log \dfrac{C_1}{C_2}$, where C_1 is the concentration of hydrogen ions inside the gelatin solution and C_2 the hydrogen ion concentration outside the gelatin solution. Hence we may substitute the value pH inside minus pH outside for the value $\log \dfrac{C_1}{C_2}$. (The term pH inside means the pH in the gelatin solution and the term pH outside means the pH in the outside solution with which the gelatin solution is in equilibrium.) This paper intends to show that if we multiply

[5] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 247.

the value of pH inside minus pH outside by 59 we can calculate with a fair degree of accuracy the P.D. observed at 24°C. in millivolts when equilibrium between the gelatin chloride solution and the outside solution is established.

II. METHODS.

The potential differences were determined with the aid of a Compton electrometer giving a deviation of about 2 mm. on the scale for 1 millivolt at a distance of about 2 m. The gelatin solution was inside the collodion bag closed with a rubber stopper through which a funnel was introduced; the funnel was filled high enough with liquid to permit the electrode to dip into the gelatin solution. The collodion bag containing the solution of gelatin-acid salt (*e.g.*, gelatin chloride) dipped into the water with which the gelatin solution was in equilibrium. The water, therefore, contained always free acid and in certain experiments also salt solution when the nature of the experiment demanded this. The second electrode was introduced into this outside watery solution. Calomel electrodes with saturated KCl solution were used and the saturated KCl solutions were brought into contact with the outside and inside solutions through glass tubes. The ends of these tubes which dipped into the inside and outside solutions were drawn out into capillaries and bent upwards to prevent the influence of gravity on the diffusion of the saturated KCl solution into the inside and outside solutions. The only potential differences existing in this system were those on the opposite sides of the membrane.

III. *The Influence of Neutral Salts on the Potential Difference between Gelatin Chloride Solutions and Outside Solutions.*

1 gm. of isoelectric gelatin was made into a 1 per cent solution by either dissolving it in H_2O or in a solution of NaCl differing in molecular concentration from M/4,096 to 1 M. To every solution so much HCl was added that the pH of the solution was 3.5. Collodion bags of a volume of about 50 cc. were filled with this solution, each collodion bag being connected with a glass tube serving as manometer, as described in preceding publications. These collodion bags

were dipped into beakers containing 350 cc. of HCl solution of pH 3.0. The HCl solutions in the beakers were made up in NaCl solutions of different concentrations and the concentration of the NaCl solution in the beaker was at the beginning of the experiment always identical with the concentration of the NaCl in the gelatin solution inside the collodion bag. The final measurements were made after 18 hours when the osmotic and the membrane equilibria were established. The osmotic pressure was a maximum (about 425 mm. water) in the gelatin chloride solution free from salt, and the osmotic pressure was the lower the higher the concentration of the salt added. This effect is represented in the upper curve of Fig. 1. The abscissae are the concentration of the NaCl solution and the ordinates the osmotic pressure. The curve shows that the osmotic pressure drops very rapidly with the increase in the concentration of NaCl.

The potential differences at the boundary of the inside and outside solutions were measured with a Compton electrometer as described and the values found are plotted on the second upper curve in Fig. 1. The scale for the ordinates was selected in such a way as to make the osmotic pressure ordinate and the ordinate for the P.D. coincide for a M/4,096 NaCl solution. The reader will notice that the two curves for osmotic pressure and P.D. coincide practically throughout which signifies that the P.D. and the osmotic pressure of the gelatin chloride solution undergo a similar depression under the influence of a neutral salt like NaCl.

We stated in the last paper that the observed P.D. is always equal to the P.D. calculated on the assumption that at equilibrium the P.D. is due to the difference in the pH inside and outside the collodion bag. The pH inside and outside were measured electrometrically and the results are contained in Table I.

On the assumption stated above we can calculate the P.D. in millivolts by multiplying the value pH inside minus pH outside by 59, and the values so obtained should agree with the observed P.D. Table II shows that this is true.

The two lower curves of Fig. 1 give the depressing effect of different concentrations of Na_2SO_4 on the osmotic pressure and on the P.D. of a 1 per cent gelatin chloride solution of pH 3.5. Everything was the same as in the preceding experiment, except that Na_2SO_4 was sub-

stituted for NaCl.   The two lower curves in Fig. 1 show that the
depressing effect of Na_2SO_4 on the osmotic pressure and the P.D. of a
gelatin chloride solution is very similar and, moreover, that the
depressing effect of Na_2SO_4 on both properties is more than twice (in
reality nearly eight times) as great as that of NaCl.   This influence
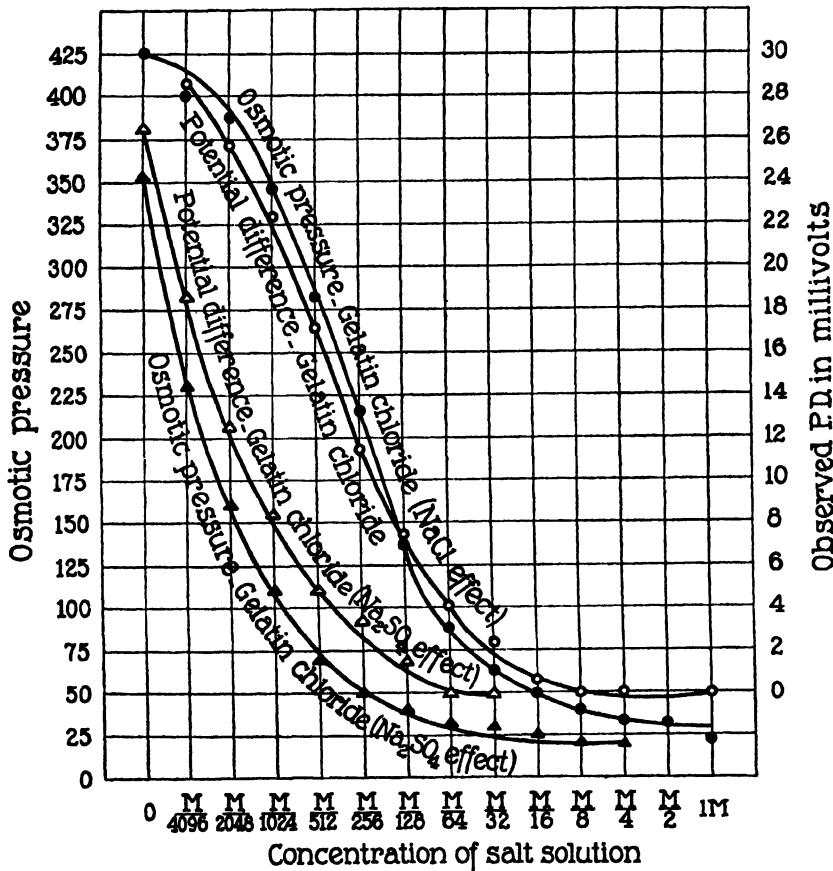
FIG. 1. Depressing effect of NaCl and Na_2SO_4 on osmotic pressure and P.D.
of gelatin chloride solutions.   Abscissae represent the concentration of salt solu-
tion, ordinates the osmotic pressure and P.D. in millivolts respectively.   The
depressing effect of neutral salts on osmotic pressure and P.D. is similar but not
identical.

TABLE I.

Influence of Concentration of NaCl on pH Inside Minus pH Outside at Equilibrium.

	Concentration of NaCl.													
	0	m/4,096	m/2,048	m/1,024	m/512	m/256	m/128	m/64	m/32	m/16	m/8	m/4	m/2	1 m
pH inside.......	3.60	3.55	3.52	3.46	3.41	3.36	3.31	3.27	3.22	3.30	3.32	3.32	3.28	3.19
pH outside.....	3.09	3.08	3.09	3.11	3.13	3.16	3.20	3.20	3.18	3.28	2.30	3.30	3.26	3.19
pH inside minus pH outside...	0.51	0.47	0.43	0.35	0.28	0.20	0.11	0.07	0.04	0.02	0.02	0.02	0.02	0.00

TABLE II.[6]

Depressing Effect of NaCl on P.D. at Equilibrium.

Molecular concentration of NaCl.	P. D. observed.	P. D. calculated.
	millivolts	*millivolts*
0		+29.9
m/4,096	+28.6	+27.6
m/2,048	+25.7	+25.2
m/1,024	+22.3	+20.6
m/512	+17.1	+16.4
m/256	+11.4	+11.7
m/128	+ 7.4	+ 6.5
m/64	+ 4.0	+ 4.1
m/32	+ 2.3	+ 2.3
m/16	+ 0.6	+ 1.2
m/8	0.0	+ 1.2
m/4	0.0	+ 1.2
m/2	− 1.7	+ 1.2
1 m	0.0	0.0

[6] The sign of the observed P.D. was apparently, but not in reality, the reverse of the sign of the calculated P.D. This was due to the difference in the arrangement of the hydrogen electrodes in both cases. In the "observed" P.D. the membrane (serving as a hydrogen electrode) was between the concentrated and dilute HCl, while in the "calculated" values the P.D. was obtained from the potentiometric determinations of the pH. In this latter case the two hydrogen electrodes were separated by a concentrated and a dilute solution. The "observed" P.D. was hence between two solutions of different concentrations while in the "calculated" values we measured the P.D. between two electrodes. In our tables the apparent (but not real) reversal of sign due to the difference in arrangement of the hydrogen electrodes in the two cases is corrected.

of the valency of anion in the case of gelatin-acid salt has been discussed in preceding papers. The question now arises: Does the value of the difference in the pH inside minus pH outside vary similarly as the values of the observed P.D. and the observed osmotic pressure, and do we get values approximating the observed P.D. if we multiply the value pH inside minus pH outside by 59? Table III gives the values for pH inside and outside and also the difference, pH inside minus pH outside, at the point of equilibrium (*i.e.*, after 18 hours).

The values for the P.D. calculated by multiplying the values for pH inside minus pH outside by 59 agree remarkably well with the observed values for P.D.

TABLE III.

Influence of Concentration of Na_2SO_4 on pH Inside Minus pH Outside at Equlibrium.

	Concentration of Na_2SO_4.											
	0	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4
pH inside	3.54	3.41	3.35	3.32	3.29	3.30	3.33	3.38	3.41	3.41	3.37	3.29
pH outside	3.07	3.12	3.14	3.17	3.20	3.24	3.30	3.35	3.38	3.38	3.36	3.28
pH inside minus pH outoutside	0.47	0.29	0.21	0.15	0.09	0.06	0.03	0.03	0.03	0.03	0.01	0.01

We will consider as a third case the influence of a salt of the type $CaCl_2$ on the osmotic pressure and the P.D. of a gelatin chloride solution. It had been shown in a preceding paper that the depressing effect of $CaCl_2$ is always about twice as great as that of an equimolecular concentration of NaCl and that hence the whole effect of $CaCl_2$ is due exclusively to the Cl ion. Fig. 2 shows that the depressing effects of $CaCl_2$ on the osmotic pressure and the P.D. run closely parallel and that the depressing effect of $CaCl_2$ on P.D. is about the same as that of a NaCl of twice the molecular concentration. Table V gives the influence of $CaCl_2$ on the values of pH inside minus pH outside, and shows that the agreement between the observed and the calculated P.D. is quite close.

It follows from these experiments, first, that the curves representing the influence of neutral salts on P.D. are similar to the curves representing the influence of the same salts on the osmotic pressure of
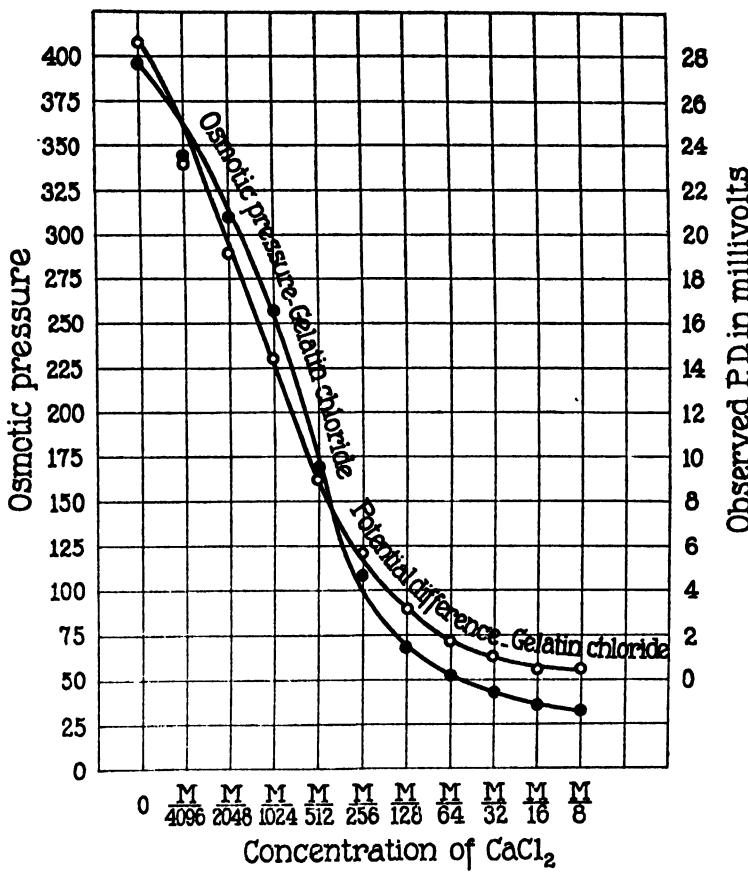
FIG. 2. Similarity of depressing effect of CaCl₂ on osmotic pressure and P.D. of gelatin chloride solution.

the same gelatin chloride solutions; and second, that the P.D. calculated by multiplying the values of pH inside minus pH outside by 59 agrees quite closely with the observed P.D.

TABLE IV.

Depressing Effect of Na₂SO₄ on P.D. at Equilibrium.

Molecular concentration of Na₂SO₄.	P. D. observed.	P. D. calculated.
	millivolts	*millivolts*
0	+26.5	+27.6
M/4,096	+18.6	+17.0
M/2,048	+12.5	+12.3
M/1,024	+ 8.4	+ 8.8
M/512	+ 4.7	+ 5.3
M/256	+ 3.4	+ 3.5
M/128	+ 1.5	+ 1.7
M/64	0.0	+ 1.7
M/32	0.0	+ 1.7
M/16	0.0	+ 1.7
M/8	0.0	+ 0.6
M/4	0.0	+ 0.6

TABLE V.

Influence of Concentration of CaCl₂ on pH Inside Minus pH Outside and on P.D. at Equilibrium.

	Concentration of CaCl₂.										
	0	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8
pH inside.....	3.55	3.45	3.41	3.36	3.30	3.28	3.26	3.25	3.25	3.25	3.22
pH outside....	3.05	3.06	3.09	3.12	3.15	3.17	3.20	3.22	3.24	3.24	3.22
pH inside minus pH outside........	0.50	0.39	0.32	0.24	0.15	0.11	0.06	0.03	0.01	0.01	0.0
P.D. calculated (millivolts)..	+29.5	+23.0	+18.9	+14.1	+8.8	+6.5	+3.5	+1.8	+0.6	+0.6	0.0
P.D. observed (millivolts)..	+28.6	+23.4	+19.2	+14.5	+9.1	+5.7	+3.1	+1.8	+1.1	+0.5	+0.5

IV. *Influence of Neutral Salts on the Potential Differences between Solid Gelatin and Outside Solution.*

It is well known that the addition of neutral salt depresses the swelling of solid gelatin chloride or of any gel with a pH different from that of the isoelectric point. This is intelligible on the basis of

Procter's osmotic theory of swelling which assumes that the swelling of a gel is due to the osmotic pressure inside the gel, the surface of the gel acting as a membrane impermeable to gelatin ions but permeable to the H or Cl or salt ions. On this assumption we should expect the establishment of a Donnan equilibrium between the liquid inside the gel and the outside liquid, and Procter has proved that this is the case. We should also expect a P.D. at the surface of the gel between the solution inside and outside the gel, due to the Donnan equilibrium, and we should, moreover, expect that the P.D. could be calculated from the value of pH inside minus pH outside as described for the experiments on osmotic pressure. This expectation is confirmed except that the results are liable to be less regular than in the case of the osmotic pressure experiments. This is probably due to accidental errors, one being possibly that some of the gel dissolves in the outside solution so that the outside solution is no longer free from gelatin. Such an error does not exist when we separate a gelatin solution from the outside solution by a collodion membrane.

Our method was as follows. 1 gm. of powdered gelatin of pH 7.0 was brought to the isoelectric point by treatment with M/128 acetic acid and subsequent washing with cold H_2O as described in previous papers. The doses of powdered wet isoelectric gelatin were then put into 200 cc. of M/128 HCl made up in different concentrations of NaCl varying from 0 to M/8, and the degree of swelling was measured in terms of the height of a column of the powdered gelatin in 100 cc. graduates after equilibrium was established (after two hours). From this the volume of the isoelectric gelatin was deducted. Fig. 3 shows that the swelling diminished with the quantity of salt added. The mass was put on a filter and allowed to drain thus separating the gelatin from the supernatant liquid. The gelatin was then melted and its pH determined electrometrically. This gave us the pH inside, and by determining the pH of the outside solution the values of the pH inside minus pH outside were ascertained. The liquid gelatin was then poured into cylinders with a small bent side tube attached, which was also filled with gelatin. The gelatin was then cooled and allowed to set to a jelly. The P.D. between the solid jelly and the outside solution was then determined by pushing one electrode into the gel while the other electrode was introduced into the

beaker containing the outside solution. The latter solution was brought into contact with the gelatin in the side tube, the latter
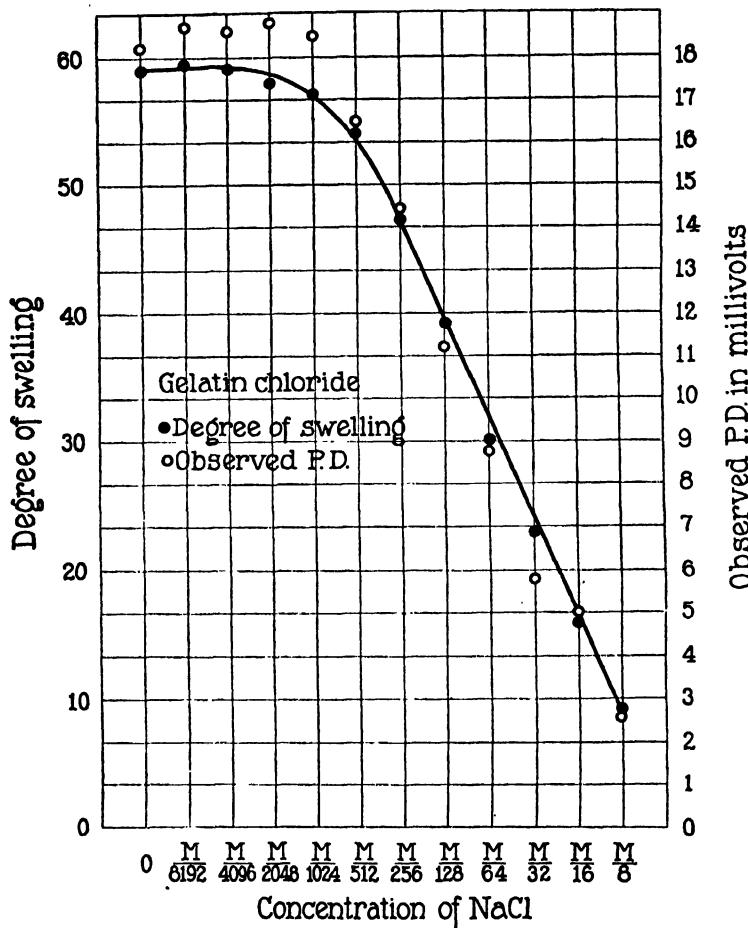
FIG. 3. Similarity of depressing effect of NaCl on swelling and P.D. of solid gelatin chloride.

dipping into the beaker. Table VI gives a comparison of the observed P.D. and those calculated by multiplying the values pH inside minus pH outside by 59. The agreement is satisfactory.

TABLE VI.

Influence of Concentration of NaCl on pH Inside Minus pH Outside and on P.D. of Solid Gelatin Chloride at Equilibrium.

	Concentration of NaCl.											
	0	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8
pH inside..............	2.71	2.70	2.69	2.69	2.70	2.69	2.65	2.61	2.57	2.56	2.54	2.50
pH outside............	2.38	2.38	2.37	2.37	2.38	2.38	2.39	2.40	2.42	2.43	2.45	2.46
pH inside minus pH outside....	0.33	0.32	0.32	0.32	0.32	0.31	0.26	0.21	0.15	0.13	0.09	0.04
P.D. calculated (millivolts)........	+19.4	+18.8	+18.8	+18.8	+18.8	+18.2	+15.3	+12.4	+8.8	+7.6	+5.3	+2.4
P.D. observed (millivolts)........	+18.2	+18.7	+18.6	+18.8	+18.5	+16.4	+14.3	+11.2	+8.8	+5.8	+5.0	+2.6

The same experiment was repeated with Na_2SO_4 as a salt instead of with NaCl. A comparison of Fig. 4 with Fig. 3 shows that Na_2SO_4 depresses the swelling of gelatin chloride more powerfully than NaCl
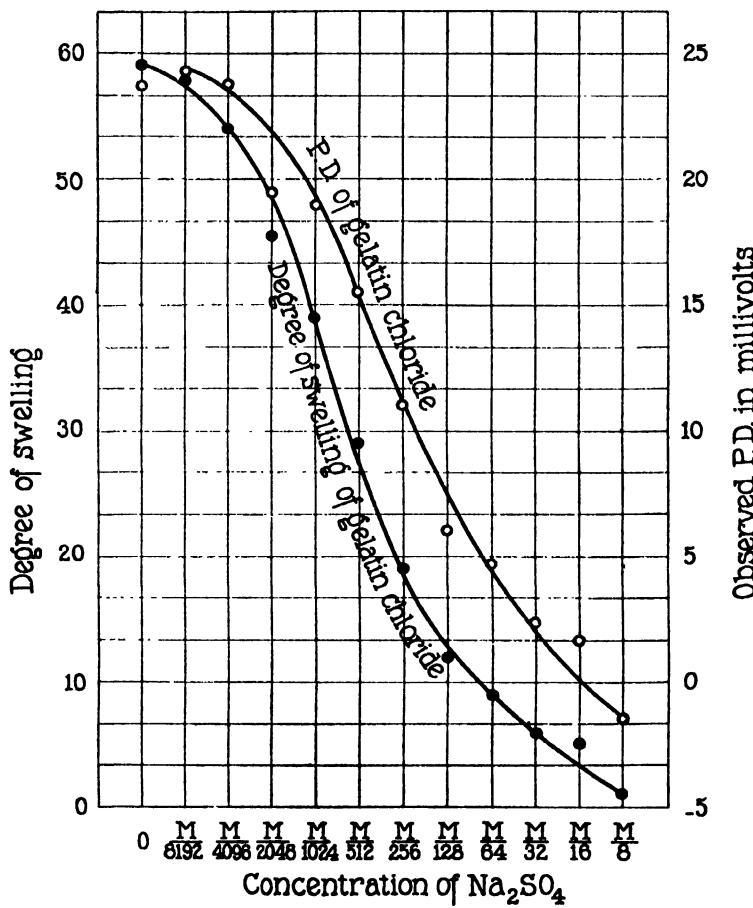
FIG. 4. Similarity of depressing effect of Na_2SO_4 on swelling and P.D. of solid gelatin chloride.

and that it depresses also the P.D. more powerfully than is done by NaCl. Table VII gives the pH inside minus pH outside, and a comparison of the P.D. calculated and observed. The agreement is again satisfactory.

TABLE VII.

Influence of Concentration of Na₂SO₄ on pH Inside Minus pH Outside and on P.D. of Solid Gelatin Chloride at Equilibrium.

	Concentration of Na₂SO₄.											
	0	$m/8{,}192$	$m/4{,}096$	$m/2{,}048$	$m/1{,}024$	$m/512$	$m/256$	$m/128$	$m/64$	$m/32$	$m/16$	$m/8$
pH inside............	2.69	2.69	2.70	2.70	2.70	2.67	2.67	2.67	2.68	2.75	2.82	2.88
pH outside...........	2.40	2.38	2.37	2.38	2.42	2.43	2.48	2.53	2.61	2.71	2.82	2.92
pH inside minus pH outside...	0.29	0.31	0.33	0.32	0.28	0.24	0.19	0.14	0.07	0.04	0.00	−0.04
P.D. calculated (millivolts)...	+17.0	+18.3	+19.5	+19.0	+16.5	+14.1	+11.2	+8.2	+4.1	+2.5	0.0	−2.5
P.D. observed (millivolts)...	+23.7	+24.3	+23.7	+19.5	+19.0	+15.5	+11.0	+6.0	+4.8	+2.4	+1.8	−1.5

We therefore come to the conclusion that the depressing influence of the neutral salts on the P.D. between solid gelatin and a surrounding aqueous solution runs parallel to the depressing effect of the same salts on the swelling of gelatin and that the P.D. can be calculated with the aid of Nernst's formula, on the assumption that the difference in the hydrogen ion concentration inside the gel and in the outside solution determines the P.D.

V. The Influence of the Hydrogen Ion Concentration of Gelatin Solutions on the P.D.

The osmotic pressure of 1 per cent solutions of originally isoelectric ' gelatin varies with the pH of the gelatin solution and with the valency of the ion in combination with the gelatin. This is illustrated in Fig. 5 where the ordinates represent the observed osmotic pressures of 1 per cent solutions of gelatin chloride, gelatin phosphate, gelatin oxalate, and gelatin sulfate. The osmotic pressure rises steeply as soon as the pH becomes less than 4.7, reaching a maximum at pH of about 3.6, and then drops steeply with a further decline of pH. Moreover, it is obvious that the curves for gelatin chloride and phosphate, both possessing a monovalent anion, are identical, that the curve for gelatin oxalate, which has mainly a monovalent anion at the pH under discussion, is almost but not quite as high as that for gelatin chloride, but that the curve for gelatin sulfate (possessing a bivalent anion) is only about half as high as that for gelatin chloride. The P.D. of 1 per cent solutions of these four salts contained in collodion bags were measured against outside aqueous solutions (without gelatin) after equilibrium was reached (i.e. after about 18 hours). The bags contained about 50 cc. of the gelatin solution while the beaker contained 350 cc. H$_2$O with so much acid that the pH of the water was at the beginning of the experiment always identical with the pH of the gelatin solution; and for the outside solution the same acid was used as for the gelatin solution. Fig. 6 gives the curves for the value of the P.D. observed. The following points of similarity between the two sets of curves for osmotic pressure (Fig. 5) and P.D. (Fig. 6) are noticeable. Both sets of curves rise from the isoelectric point with a lowering of the pH until they reach a maximum; this maxi-

mum is, however, not identical. For P.D. it varies between 3.6 and
4.0, while for osmotic pressure it lies near 3.6.[7] With a further fall
in pH both sets of curves show approximately the same steep drop.
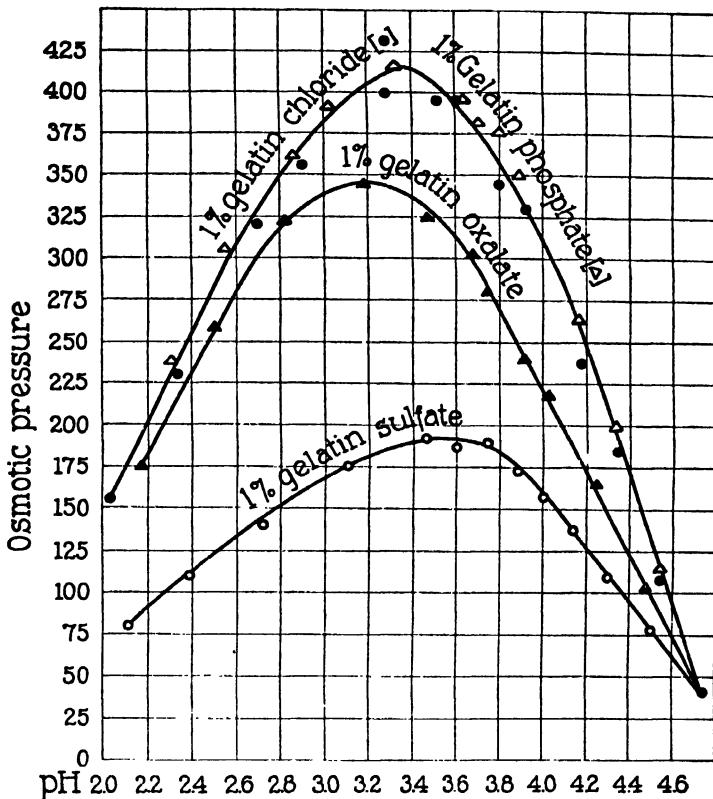
FIG. 5. Influence of pH and valency of anion on osmotic pressure of solutions
of different gelatin-acid salts.

The second point of similarity is the influence of valency. The
curves for P.D. (Fig. 6) are practically the same for gelatin chloride
and gelatin phosphate, while the curve for P.D. is considerably lower
in the case of gelatin sulfate.

[7] It may be stated incidentally that the maximum for the viscosity of gelatin
solution also lies at a different pH, namely 3.0, from the maximum for osmotic
pressure, which lies at pH 3.6.

These experiments offer an excellent opportunity to test our theory that the P.D. can be calculated with a fair degree of accuracy from the values of pH inside minus pH outside on the basis of Nernst's formula. Tables VIII, IX, and X show that this is true. The upper
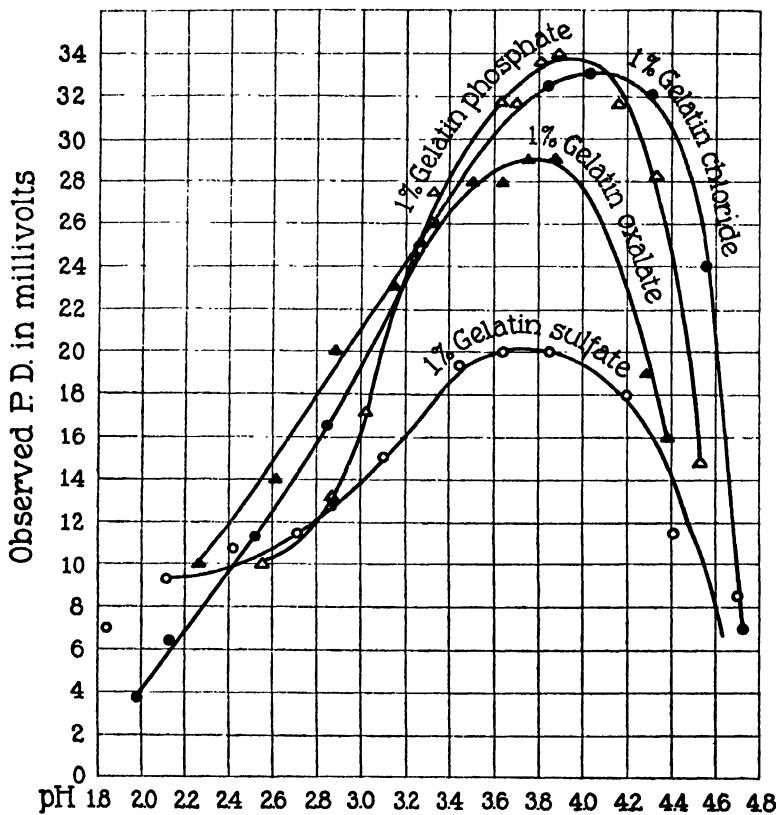
FIG. 6. Influence of pH and valency of anion on P.D. of solutions of different gelatin-acid salts. The curves in Fig. 6 are similar to (but not identical with) those in Fig. 5.

two horizontal rows give the pH inside and outside, the third horizontal row gives the difference pH inside minus pH outside, and the fourth row gives the P.D. calculated in millivolts by multiplying the values pH inside minus pH outside by 59. The last horizontal row

TABLE VIII.

Influence of the Hydrogen Ion Concentration on pH Inside Minus pH Outside and on the P.D. of Gelatin Chloride Solutions of Equilibrium.

	Cc. 0.1 N HCl added to 100 cc. 1 per cent isoelectric gelatin.											
	1	2	4	6	8	10	12.5	15	20	30	40	50
pH inside..........	4.56	4.31	4.03	3.85	3.33	3.25	2.85	2.52	2.13	1.99	1.79	1.57
pH outside.........	4.14	3.78	3.44	3.26	2.87	2.81	2.53	2.28	2.00	1.89	1.72	1.53
pH inside minus pH outside....	0.42	0.53	0.59	0.59	0.46	0.44	0.32	0.24	0.13	0.10	0.07	0.04
P.D. calculated (millivolts)........	+24.7	+31.0	+34.5	+34.5	+27.0	+25.8	+18.8	+14.0	+7.6	+5.9	+4.1	+2.3
P.D. observed (millivolts).........	+24.0	+32.0	+33.0	+32.5	+26.0	+24.5	+16.5	+11.2	+6.4	+4.8	+3.7	+2.1

TABLE IX.

Influence of the Hydrogen Ion Concentration on pH Inside Minus pH Outside and on P.D. of Gelatin Phosphate Solutions at Equilibrium.

	Cc. M/10 H_3PO_4 added to 100 cc. 1 per cent isoelectric gelatin.													
	0	1	2	4	6	7	8	10	12.5	15	20	30	40	50
pH inside..........	4.79	4.54	4.31	3.98	3.68	3.56	3.38	3.24	3.02	2.67	2.42	2.12	1.92	1.74
pH outside.........	4.70	4.10	3.77	3.40	3.14	3.04	2.90	2.80	2.66	2.39	2.22	1.98	1.83	1.67
pH inside minus pH outside.....	0.09	0.44	0.54	0.58	0.54	0.52	0.48	0.44	0.36	0.28	0.20	0.14	0.09	0.07
P.D. calculated (millivolts)........	+5.3	+25.8	+31.7	+34.0	+31.7	+30.5	+28.0	+25.8	+21.2	+16.4	+11.7	+8.2	+5.3	+4.1
P.D. observed (millivolts)..........	+5.7	+27.0	+29.0	+30.0	+30.6	+29.6	+26.5	+24.4	+22.3	+17.7	+15.6	+11.4	+9.9	+7.3

TABLE X.

Influence of the Hydrogen Ion Concentration on pH Inside Minus pH Outside and on P.D. of Gelatin Sulfate Solutions at Equilibrium.

	0	1	2	4	6	7	8	10	12.5	15	20	30	40	50
					Cc. 0.1 N H₂SO₄ added to 100 cc. 1 per cent isoelectric gelatin.									
pH inside.........	4.76	4.52	4.34	3.98	3.73	3.49	3.41	3.12	2.78	2.47	2.16	2.06	1.84	1.57
pH outside.........	4.61	4.20	3.99	3.60	3.38	3.18	3.14	2.88	2.61	2.35	2.09	2.00	1.80	1.54
pH inside minus pH outside.....	0.15	0.32	0.35	0.38	0.35	0.31	0.27	0.24	0.17	0.12	0.07	0.06	0.04	0.03
P.D. calculated (millivolts)........	+8.8	+18.8	+20.5	+22.2	+20.5	+18.1	+15.8	+14.0	+10.0	+7.0	+4.1	+3.5	+2.4	+1.8
P.D. observed (millivolts).........	+6.3	+16.3	+18.4	+19.0	+19.0	+17.4	+15.8	+13.7	+10.5	+8.4	+7.4	+5.8	+4.7	+3.7

gives the observed P.D. in millivolts. The agreement between observed and calculated P.D. is sufficiently close.

VI. *Hydrogen Ion and Chlorine Ion Potentials.*

The equation for the equilibrium condition between gelatin chloride solution and water is as stated above,

$$x^2 = y (y + z)$$

where x is the concentration of H and Cl ions in the outside solution, y the concentration of the H and Cl ions of the free HCl inside the gelatin chloride solution, and z the concentration of the Cl ions held by the gelatin.

If we write this equation in the form

$$\frac{y}{x} = \frac{x}{y + z}$$

$\frac{y}{x}$ is the ratio of hydrogen ion concentration inside over the hydrogen ion concentration outside; and $\frac{x}{y + z}$ is the ratio of the concentration of the chlorine solution outside over the chlorine solution inside. Since

$$\log \frac{y}{x} = \text{pH inside minus pH outside}$$

and

$$\log \frac{x}{y + z} = \text{pCl outside minus pCl inside}$$

it follows that

$$\text{pH inside minus pH outside} = \text{pCl outside minus pCl inside}$$

It was easy to put this consequence of Donnan's theory to a test and some of the experiments described in the preceding chapter were selected for this purpose. Inside the collodion bag was a 1 per cent solution of gelatin chloride of different pH, outside water. After 18 hours equilibrium was established between inside and outside solutions and the pH as well as the pCl were ascertained. The pCl

was determined in two different ways in the two experiments; in one experiment it was determined with the potentiometer, in the other it was determined in the gelatin chloride solution by titration with NaOH according to the method described in a preceding paper.[8] Both methods of determining the pCl led to the result that the values pCl outside minus pCl inside were for the same solution at the point of equilibrium equal to the value pH inside minus pH outside (within the limits of accuracy of the experiments). The pCl outside was identical with the pH outside, since the outside solution contained only free HCl. The values of pH were all determined potentiometrically.

TABLE XI.

Experiment 1. pCl determined by titration.									
pH of gelatin chloride solution at equilibrium									
4.13	3.69	3.30	3.10	2.92	2.78	2.46	2.26	2.01	1.76
pH inside minus pH outside									
0.56	0.58	0.50	0.49	0.44	0.44	0.33	0.23	0.15	0.10
pCl outside minus pCl inside									
0.48	0.51	0.59	0.44	0.44	0.38	0.35	0.22	0.15	0.11

Experiment 2. pCl determined electrometrically.									
pH of gelatin chloride solution at equilibrium									
4.04	3.92	3.78	3.61	3.46	3.16	2.73	2.36	2.04	1.73
pH inside minus pH outside									
0.60	0.62	0.66	0.55	0.50	0.43	0.30	0.20	0.12	0.07
pCl outside minus pCl inside									
0.55	0.60	0.57	0.50	0.53	0.38	0.32	0.17	0.12	0.07

Nernst's formula leads therefore to the same theoretical P.D. regardless of the fact whether we calculate the P.D. on the basis of the difference pH inside minus pH outside or pCl outside minus pCl inside. It is also obvious that both assumptions lead to the same sign of charge of the gelatin chloride solution. If we assume that the P.D. is determined by differences in the hydrogen ion concentration the outside solution is concentrated and the inside solution dilute; if the P.D. is determined by differences in the concentration of the Cl ions the inside solution is concentrated and the outside solution dilute. Since the common ion is positive in the former and negative in the latter case, the gelatin solution becomes positive in both cases.

[8] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 559.

outside solution.			inside solution.
$- H^+$ concentrated	membrane		H^+ dilute $+$
$- Cl^-$ dilute			Cl^- concentrated $+$

The facts of this last chapter prove beyond doubt that the equation $x^2 = y \, (y + z)$ is the correct expression for the Donnan membrane equilibrium between acid-salts of proteins with monovalent anion and water. The close analogy between the variation of the membrane potentials and the other osmotic properties of solutions of protein-acid salts, such as osmotic pressure, viscosity, and swelling, suggests that an attempt be made to derive the variations of these latter properties directly from the equation for the Donnan equilibrium. This has already been done by Procter for the swelling of gelatin chloride.

SUMMARY.

1. It is shown that a neutral salt depresses the potential difference which exists at the point of equilibrium between a gelatin chloride solution contained in a collodion bag and an outside aqueous solution (without gelatin). The depressing effect of a neutral salt on the P.D. is similar to the depression of the osmotic pressure of the gelatin chloride solution by the same salt.

2. It is shown that this depression of the P.D. by the salt can be calculated with a fair degree of accuracy on the basis of Nernst's logarithmic formula on the assumption that the P.D. which exists at the point of equilibrium is due to the difference of the hydrogen ion concentration on the opposite sides of the membrane.

3. Since this difference of hydrogen ion concentration on both sides of the membrane is due to Donnan's membrane equilibrium this latter equilibrium must be the cause of the P.D.

4. A definite P.D. exists also between a solid block of gelatin chloride and the surrounding aqueous solution at the point of equilibrium and this P.D. is depressed in a similar way as the swelling of the gelatin chloride by the addition of neutral salts. It is shown that the P.D. can be calculated from the difference in the hydrogen ion concentration inside and outside the block of gelatin at equilibrium.

5. The influence of the hydrogen ion concentration on the P.D. of a gelatin chloride solution is similar to that of the hydrogen ion concentration on the osmotic pressure, swelling, and viscosity of gelatin solutions, and the same is true for the influence of the valency of the anion with which the gelatin is in combination. It is shown that in all these cases the P.D. which exists at equilibrium can be calculated with a fair degree of accuracy from the difference of the pH inside and outside the gelatin solution on the basis of Nernst's logarithmic formula by assuming that the difference in the concentration of hydrogen ions on both sides of the membrane determines the P.D.

6. The P.D. which exists at the boundary of a gelatin chloride solution and water at the point of equilibrium can also be calculated with a fair degree of accuracy by Nernst's logarithmic formula from the value pCl outside minus pCl inside. This proves that the equation $x^2 = y (y + z)$ is the correct expression for the Donnan membrane equilibrium when solutions of protein-acid salts with monovalent anion are separated by a collodion membrane from water. In this equation x is the concentration of the H ion (and the monovalent anion) in the water, y the concentration of the H ion and the monovalent anion of the free acid in the gelatin solution, and z the concentration of the anion in combination with the protein.

7. The similarity between the variation of P.D. and the variation of the osmotic pressure, swelling, and viscosity of gelatin, and the fact that the Donnan equilibrium determines the variation in P.D. raise the question whether or not the variations of the osmotic pressure, swelling, and viscosity are also determined by the Donnan equilibrium.

DONNAN EQUILIBRIUM AND THE PHYSICAL PROPERTIES OF PROTEINS.

II. Osmotic Pressure.

By JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, March 23, 1921.)

I. INTRODUCTION.

It was pointed out in two preceding papers[1] that the curves representing the influence of electrolytes on the membrane potentials between gelatin solutions and water at the point of equilibrium show a marked similarity to the curves representing the influence of the same electrolytes on osmotic pressure, swelling, and viscosity of gelatin. There was no doubt left that the Donnan equilibrium accounted quantitatively for these variations in the case of membrane potentials[2] and the question therefore arose whether the same equilibrium condition can account also for the corresponding variations of the osmotic pressure of protein solutions. An attempt was, therefore, made to calculate the osmotic pressures on the basis of the Donnan equilibrium in order to find out whether the curves for the calculated values would show the characteristics of the curves representing the observed values for osmotic pressures. The depressing effect of the addition of a neutral salt on the osmotic pressure of colloidal solutions was predicted by Donnan and offered no theoretical difficulty.[3] The real test was whether the Donnan equilibrium would be able to account for the peculiar curves obtained when the observed osmotic pressures of 1 per cent solutions of gelatin-acid salts are plotted as a function of the pH.

---

[1] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 557, 667.

[2] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 667.

[3] Donnan, F. G., *Z. Elektrochem.*, 1911, xvii, 572. Donnan, F. G., and Harris, A. B., *J. Chem. Soc.*, 1911, xcix, 1554. Donnan, F. G., and Garner, W. E., *J. Chem. Soc.*, 1919, cxv, 1313.

Fig. 1 shows a set of such curves where the abscissæ are the pH of the gelatin solution at equilibrium, and the ordinates the observed osmotic pressures. There are two outstanding peculiarities in these curves, namely, first, that they all rise from a minimum at pH 4.7 (the isoelectric point of gelatin) until they reach a maximum at pH
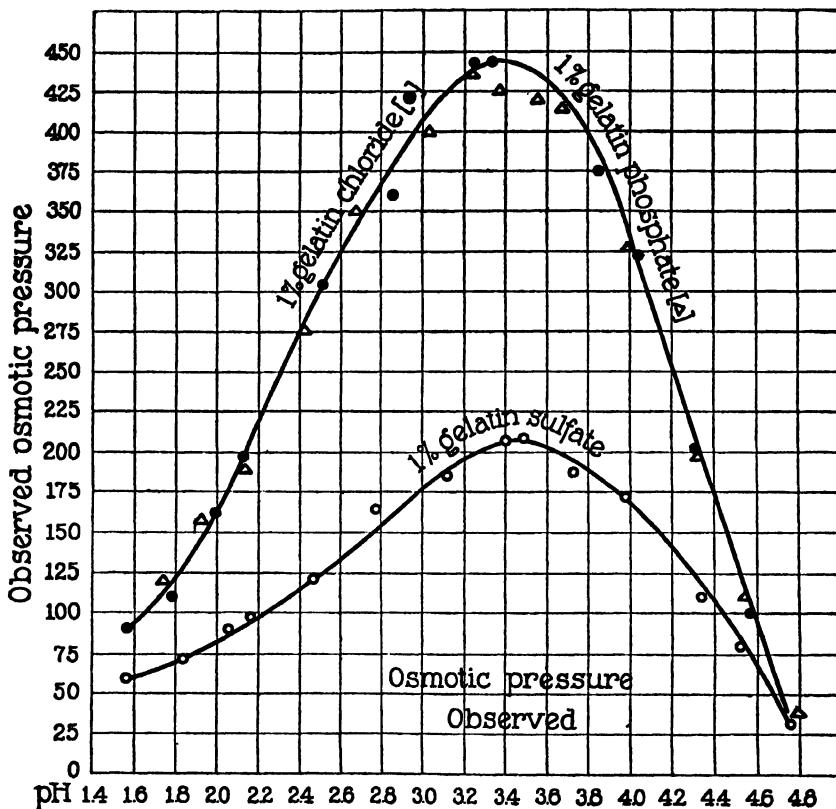
FIG. 1. Curves representing the influence of pH and valency of anion on osmotic pressure of solutions of gelatin-acid salts containing 1 gm. of originally isoelectric gelatin in 100 cc. solution. The curves for gelatin chloride and gelatin phosphate are identical since the anions, Cl and H_2PO_4, of these two gelatin salts are mono-valent. The curve for gelatin sulfate is less than half as high as the curve for the two other salts because the anion of gelatin sulfate is bivalent. Both curves rise from the isoelectric point at 4.7 to a maximum at pH about 3.4 or 3.5, and then drop rapidly again.

about 3.5, and then drop again; and second, that only the valency of the ion in combination with a protein influences its osmotic pressure (or degree of swelling, etc.), while the specific nature of the ion aside from its valency has no influence. The latter fact is really the crucial point which decides between colloid chemistry and classical physical chemistry. If only the valency of the ion in combination with a protein is of importance and if gelatin-acid salts of the same pH and the same concentration of originally isoelectric gelatin have the same osmotic pressure, provided the anion of the gelatin-acid salt has the same valency, a suspicion must arise that we are dealing with some equilibrium condition for which classical physical chemistry is able to account. The writer has shown in preceding papers that gelatin chloride, nitrate, acetate, succinate, tartrate, citrate, and phosphate have at the same pH and the same concentration of originally isoelectric gelatin the same osmotic pressure; and it was shown by titration curves that the anion in all these salts is mono-valent. The titration curves show also that the anion in gelatin sulfate is bivalent and we have found that the osmotic pressure of gelatin sulfate is less than half that of gelatin chloride or phosphate at the same pH and for the same concentration of originally isoelec-tric gelatin.[4]

Fig. 1 illustrates this valency effect in the observed osmotic pres-sure. The curves for the observed osmotic pressure of gelatin chlo-ride and gelatin phosphate are identical while the curve for gelatin sulfate is considerably lower.

It is the purpose of this paper to show that we can calculate with a fair degree of accuracy ·the osmotic pressure of gelatin solutions on the assumption of the validity of Donnan's equilibrium equation and the validity of van't Hoff's theory of osmotic pressure.

II. Theoretical Data.

A gelatin chloride solution contains free hydrochloric acid, gelatin chloride (which dissociates electrolytically like any other salt in watery solution), and non-ionogenic protein molecules. A 1 per cent gelatin chloride solution of about pH 3.5 is in equilibrium with a

[4] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 85, 247, 391; *Science*, 1920, lii, 449.

HCl solution (free from protein) of a pH of about 3.0, both solutions being separated by a collodion membrane.

Let y be the concentration of the H and Cl ions of the free HCl inside a gelatin chloride solution (containing 1 gm. of originally isoelectric gelatin in 100 cc.), z the concentration of the Cl ions held by the gelatin ions, and a the sum of the gelatin ions and non-ionized molecules of gelatin. For the sake of simplification we assume complete electrolytic dissociation of the gelatin chloride and of the HCl. In this case the real osmotic pressure of the inside solution is determined by

$$2y + z + a$$

Since, however, the outside solution is at equilibrium not H_2O but a HCl solution—in the example selected of about pH 3.0—the *observed* osmotic pressure is the difference between the osmotic pressure of the inside solution against H_2O and the osmotic pressure of the outside solution.

Let x be the concentration of the H ions in the outside solution, then the osmotic pressure of the outside solution is determined by $2x$.

Hence the observed osmotic pressure P_o of the gelatin chloride solution is determined by

$$P_o = 2y + z + a - 2x$$

P_o is observed experimentally, y can be calculated from the pH inside, and x from the pH outside.

z can be calculated from Donnan's equilibrium equation in the form given it by Procter[5]

$$x^2 = y\,(y + z) \qquad (1)$$

$$z = \frac{(x + y)\ (x - y)}{y}$$

where x, y, and z have the significance stated above. We have seen in the preceding paper that this equation leads to correct results in regard to the P.D.

[5] Procter, H. R., *J. Chem. Soc.*, 1914, cv, 313. Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307.

a is unknown, and we therefore can only calculate for the present the values of

$$2y + z - 2x$$

If we express the theoretical osmotic pressure of a grammolecular solution in terms of mm. pressure of a column of H_2O we get (with correction for a temperature of 24°C.)

$$22.4 \times 760 \times 13.6 \times \frac{297}{273} = 2.5 \times 10^5$$

In other words, a theoretical pressure of 2.5 mm. H_2O corresponds to a concentration of 10^{-5} N. In the following tables all concentrations are expressed in terms of 10^{-5} N and hence we only need to multiply the values for $2y + z - 2x$ given in our tables by 2.5 to obtain the calculated osmotic pressure of the gelatin solution (neglecting the osmotic pressure of the gelatin ions and molecules).

Equation (1) holds in the case of solutions of all gelatin-acid salts with monovalent anion; i.e. gelatin chloride, acetate, phosphate, tartrate, citrate, etc. When, however, the anion of a gelatin-acid salt is divalent, as in the case of gelatin sulfate, the equilibrium equation becomes one of the third degree. If x be the hydrogen ion concentration of the outside solution, the concentration of the SO_4 ion in the outside solution becomes $\frac{x}{2}$. If y be the concentration of the H ions of the free sulfuric acid in the inside solution, $\frac{y}{2}$ is the concentration of the SO_4 ions of the free acid inside the gelatin sulfate solution. In the case of gelatin chloride z represented the concentration of chlorine ions in combination with the gelatin; hence $\frac{z}{2}$ will represent the concentration of SO_4 ions in combination with the same number of gelatin ions.

The equilibrium equation, therefore, assumes in the case of gelatin sulfate the following form

$$x^2 \cdot \frac{x}{2} = y^2 \frac{(y + z)}{2} \qquad (2)$$

From equation (2) follows

$$z = \frac{x^3 - y^3}{y^2}$$

The osmotic pressure of the gelatin solution should therefore be calculated from the following values (omitting the share of the osmotic pressure due to the gelatin)

$$\frac{3}{2} y + \frac{z}{2} - \frac{3}{2} x$$

III. The Calculated Curves.

Solutions containing 1 gm. of originally isoelectric gelatin in 100 cc. and containing different quantities of acid were prepared. Collodion bags cast in the form of Erlenmeyer flasks of 50 cc. volume were filled with the 1 per cent solution of a gelatin-acid salt and put into a beaker containing 350 cc. of H₂O. In order to accelerate the establishment of the equilibrium between inside and outside solutions a certain amount of acid was added to the outside water (e.g., HCl in the experiments with gelatin chloride, H₃PO₄ in the experiments with gelatin phosphate, etc.). Each Erlenmeyer flask was closed with a rubber stopper perforated by a glass tube serving as a manometer. All this was described in more detail in previous publications.

In Fig. 2 are plotted the values of the calculated osmotic pressures for 1 per cent solutions of gelatin chloride, gelatin phosphate, and gelatin sulfate, and Tables I, II, and III give the data on the basis of which the curves in Fig. 2 are calculated. The experiments from which these calculations were made are identical with the experiments from which the curves for the observed osmotic pressures in Fig. 1 were plotted. The abscissæ in Fig. 2 are the pH in the inside solution at the point of equilibrium, the ordinates are the values for osmotic pressure calculated on the basis of the Donnan equilibrium as discussed before. The reader will notice that the three curves plotted in Fig. 2 show not only the same qualitative characteristics as the curves for the observed osmotic pressures in Fig. 1, but show them almost quantitatively; except that a correction for the value of osmotic pressure due to the gelatin particles itself may have to be

TABLE I.

Gelatin Chloride.

Observed and Calculated Osmotic Pressures of Gelatin Chloride Containing 1 gm. of Originally Isoelectric Gelatin in 100 cc. Solution at Equilibrium.

pH inside	4.56	4.31	4.03	3.85	3.33	3.25	2.85	2.52	2.13	1.99	1.79	1.57
pH outside	4.14	3.78	3.44	3.26	2.87	2.81	2.53	2.28	2.00	1.89	1.72	1.53
$y = C_H$ inside $\times 10^6$	2.7	4.9	9.3	14.1	46.8	56.2	141	302	741	1023	1622	2692
$x = C_H$ outside $\times 10^6$	7.2	16.6	36.3	54.9	135.0	155.0	295	524	1000	1288	1905	2951
$z = \dfrac{(x+y)(x-y)}{y}$	16.5	51.4	132.5	200.0	343.0	372.0	477	608	609	600	612	544
$2y + z - 2x$	7.5	28.0	78.5	118.4	166.6	174.4	169	164	91	70	46	26
Observed osmotic pressure	100	202	322	375	443	442	360	303	198	162	110	90
Calculated osmotic pressure, neglecting osmotic pressure of protein	19	70	196	296	416	436	422	410	227	175	115	65

TABLE II.

1 Per Cent Gelatin Phosphate.

Observed and Calculated Osmotic Pressures at Equilibrium.

pH inside............	4.79	4.54	4.31	3.98	3.68	3.56	3.38	3.24	3.02	2.67	2.42	2.12	1.92	1.74
pH outside..........	4.70	4.10	3.77	3.40	3.14	3.04	2.90	2.80	2.66	2.39	2.22	1.98	1.83	1.67
$y = C_H$ inside $\times 10^6$......	1.6	2.9	4.9	10.5	20.9	27.5	41.7	57.5	95.5	213.8	380.2	758.6	1202	1820
$x = C_H$ outside $\times 10^6$......	2.0	7.9	16.9	39.8	72.4	91.2	125.9	158.5	218.8	407.4	602.6	1047.0	1479	2138
$s = \dfrac{(x+y)(x-y)}{y}$......	0.9	18.6	53.3	140.0	228.0	231.0	338.0	380.0	405.0	556.0	575.0	686.0	617	690
$2y + s - 2x$..........	0.1	8.6	31.3	81.4	125.0	103.6	169.6	178.0	158.0	169.0	130.0	109.0	63	54
Observed osmotic pressure....	34	111	199	328	416	420	426	436	401	350	275	190	158	121
Calculated osmotic pressure, neglecting osmotic pressure of protein............		22	77	203	310	258	423	445	395	420	324	273	157	135

TABLE III.

1 Per Cent Gelatin Sulfate.

Observed and Calculated Osmotic Pressure at Equilibrium.

pH inside	4.76	4.52	4.34	3.98	3.73	3.49	3.41	3.12	2.78	2.47	2.16	2.06	1.84	1.57
pH outside	4.61	4.20	3.99	3.60	3.38	3.18	3.14	2.88	2.61	2.35	2.09	2.00	1.80	1.54
$y = C_H$ inside $\times 10^6$	1.7	3.0	4.6	10.4	18.6	32.3	38.9	75.9	166	339	692	871	1445	2692
$x = C_H$ outside $\times 10^6$	3.1	6.3	10.2	25.1	41.7	66.0	72.4	131.8	245.5	447	813	1000	1585	2884
$z = \dfrac{x^3 - y^3}{y^2}$	8.3	24.7	45.8	136	191.5	243	212	322	390	435	433	449	466	620
$\dfrac{3}{2}y + \dfrac{z}{2} - \dfrac{3}{2}x$	2	7.35	14.5	46	64	71	55.8	77	77	55	37	31	23	20
Observed osmotic pressure	33	79	110	172	188	208	208	185	164	122	98	89	72	61
Calculated osmotic pressure, neglecting osmotic pressure of protein	5	18.5	36	115	160	178		192	192	138	92.5	77.5	57.5	50

added, a point which will be discussed in the next chapter. What is
of importance here is the following. The curves for osmotic pres-
sure calculated on the basis of the Donnan equilibrium and plotted
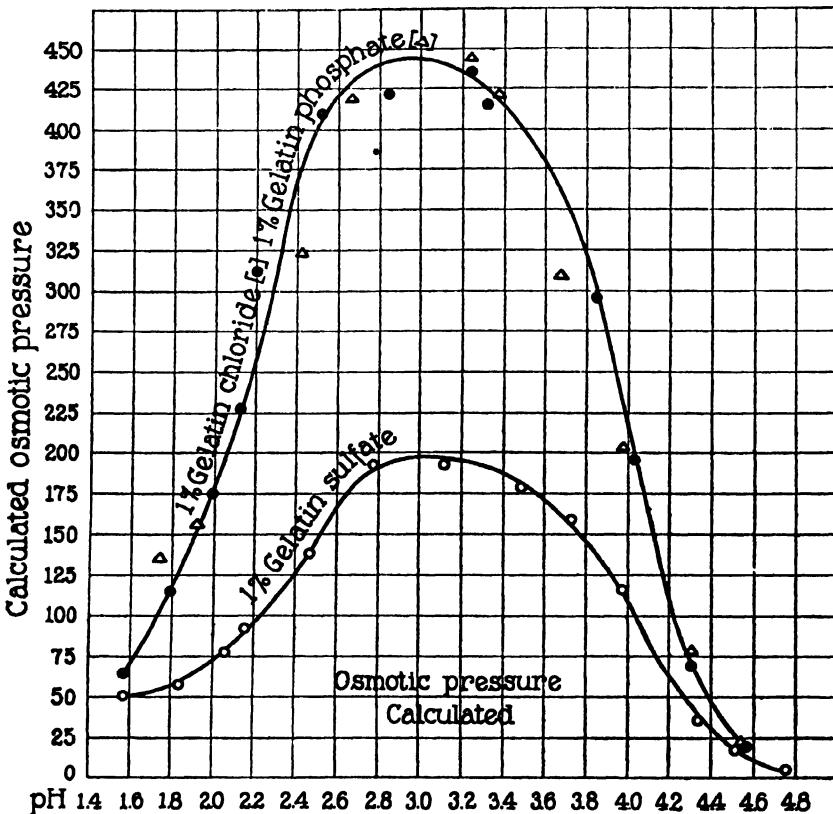
FIG. 2. Calculated curves of osmotic pressure taken from the data of the experi-
ments represented in Fig. 1. The calculation is made on the basis of the validity
of Donnan's theory of membrane equilibrium. The calculations lead to curves
resembling the curves in Fig. 1 in all essential points, in regard to valency effect
of the anion, as well as in regard to influence of pH. (See legend in Fig. 1.)

in Fig. 2 resemble the curves for the osmotic pressure observed in
the same experiments represented in Fig. 1 in the following
essential points.

1. The curve for the calculated osmotic pressure of gelatin chloride is identical with the curve for the calculated osmotic pressure of gelatin phosphate, and the same is true for the two corresponding curves representing the observed osmotic pressures (Figs. 1 and 2).

. 2. The curve for the calculated osmotic pressure of gelatin sulfate is a little less than half as high as the curves for the calculated osmotic pressures of gelatin chloride and gelatin phosphate; and the same is true for the curves representing the observed osmotic pressures of gelatin sulfate and gelatin chloride.

3. All the curves in Figs. 1 and 2 rise from a minimum at pH 4.7, reach a maximum (which lies at pH 3.4 or 3.5 for the observed, and at 3.0 for the calculated curves), and then drop again as steeply as they rose on the other side. Moreover, the absolute values of observed and calculated osmotic pressures agree almost quantitatively, a fact which will be discussed more fully in Chapter V.

It may be added that the curve for the calculated values of the osmotic pressure of gelatin oxalate solutions agrees also with the curve for the observed values of the osmotic pressure of solutions of the same gelatin salt, both being slightly lower than the curves for gelatin chloride.

We can therefore say that (with the exception of two minor discrepancies to be discussed further on) the Donnan equilibrium accounts not only qualitatively but almost quantitatively for (*a*) *the valency effect of the anion with which the gelatin is in combination; (b) for the effect of the pH.*

Thus two of the most puzzling problems of the colloid chemistry of proteins seem to find their solution on the basis of classical physical chemistry.

IV. The Presumable Osmotic Pressure of Gelatin Particles.

The question now arises what the possible share of the protein particles in the osmotic pressure may be. Different concentrations of gelatin phosphate from 2 per cent to $\frac{1}{2}$ per cent were prepared, all having a pH of 3.5. The gelatin phosphate solutions were put into Erlenmeyer flasks of 50 cc. volume, connected with a glass tube serving as a manometer as described, and these flasks were put into beakers containing 350 cc. of H_2O, the pH of which was brought at

the beginning of the experiment to 3.5 through the addition of H_3PO_4. When the bags containing gelatin phosphate solutions are put into water the latter diffuses rapidly into the gelatin solution thereby lowering the concentration of the gelatin solution. To avoid this error so much gelatin phosphate solution was poured into each bag and glass tube that at the beginning of the experiment the liquid reached already to about that level which from preceding experiments we knew the gelatin solution would reach in the manometer at the point of osmotic equilibrium. All experiments were made in duplicate. In addition to the osmotic pressure we measured the pH inside and outside after equilibrium was reached. From these latter data the osmotic pressure due to the H and PO_4 ions could be calculated, being equal to

$$(2y + z - 2x) \times 2.5 \text{ mm } H_2O.$$

By deducting this value from the observed osmotic pressure in each case it was hoped to obtain a rational value for the share of the protein particles in the observed osmotic pressure. Table IV gives the results.

The reader's attention is called to the last two rows of figures (Table IV) giving the difference between the observed and the calculated osmotic pressures, since if this difference actually represents the osmotic pressure due to the gelatin particles, the figures should be in direct proportion to the concentration of the gelatin. The experiments were all made in duplicate to give some idea of the magnitude of error and it is obvious that the error may be considerable, 25 per cent or more, because the errors in the observed and the calculated values are additive. Thus the "difference" is for $\frac{3}{4}$ per cent solution in one case 92, in the other 61, a variation of 50 per cent! If we take this into consideration we may conclude that the differences between the observed and the calculated osmotic pressures are compatible with the idea that the difference is the value for the osmotic pressure due to the gelatin particles in solution.

This would lead us to the conclusion that the osmotic pressure due to the gelatin particles in a 1 per cent solution (of originally isoelectric gelatin) of gelatin phosphate of pH 3.60 is about 100 mm. H_2O. Since the osmotic pressure of 1 grammolecule is about 250,000 mm.

TABLE IV.

Influence of Concentration of Gelatin Phosphate of pH of About 3.6 on the Osmotic Pressure. (All Experiments Were Made in Two Sets.)

	Concentration of gelatin in per cent.									
	2	2	1½	1½	1	1	½	½	¼	¼
pH inside at equilibrium	3.64	3.66	3.60	3.60	3.65	3.66	3.60	3.60	3.61	3.62
pH outside at equilibrium	3.02	3.02	3.02	3.01	3.12	3.11	3.12	3.14	3.21	3.19
$y = C_H$ inside $\times 10^6$	22.9	21.9	25.1	25.1	22.4	21.9	25.1	25.1	24.6	24.0
$x = C_H$ outside $\times 10^6$	95.5	95.5	95.5	97.7	75.9	77.6	75.9	72.4	61.7	64.6
$z = \dfrac{(x+y)(x-y)}{y}$	375	395	338	355	235	253	204	184	130	150
$2y + z - 2x$	230	248	197	210	128	142	102	89	56	69
Observed osmotic pressure	860	860	715	680	420	445	316	314	186	186
Calculated osmotic pressure (ignoring gelatin)	576	620	493	523	320	355	255	222	140	172
Difference (osmotic pressure due to gelatin)	284	240	222	157	100	90	61	92	46	14
Mean	262		190		95		73		26	

H$_2$O and since 1 liter of a 1 per cent solution of gelatin contains 10 gms. of gelatin, the molecular weight of gelatin should be expected to be in the neighborhood of 25,000. The experiment just described for gelatin phosphate was repeated for gelatin chloride, with similar results.

According to Dakin's[6] recent analyses gelatin contains 1.4 per cent phenylalanine. Since 1 molecule of gelatin cannot contain less than 1 molecule of phenylalanine and since the molecular weight of this

TABLE V.

Influence of Concentration of Albumin Chloride of pH of About 3.4 on the Osmotic Pressure.

	Concentration of Egg Albumin in per cent.					
	4	3	2	1	½	¼
pH inside at equilibrium.....	3.34	3.32	3.38	3.40	3.40	3.40
pH outside at equilibrium....	2.98	2.97	3.07	3.14	3.19	3.24
$y = C_H$ inside $\times 10^5$........	45.7	47.9	41.7	39.8	39.8	39.8
$x = C_H$ outside $\times 10^5$.......	104.7	107.2	85.1	72.4	64.5	57.5
$z = \dfrac{(x+y)(x-y)}{y}$.........	194.0	192.0	132.0	92.0	64.6	43.3
$2y + z - 2x$................	76	74	45	27	15	8
Observed osmotic pressure....	776	555 + x	375	163	75	36
Calculated osmotic pressure (ignoring albumin)........	190	185	113	67	39	20
Difference (osmotic pressure due to albumin)..........	586	370 + x	262	96	36	16

amino-acid is 165 the lowest possible molecular weight of gelatin is 11,800. If a molecule of gelatin contains two molecules of phenylalanine, the molecular weight should be about 23,600. This would be approximately the figure we might expect from the data of Table IV on the assumption that the differences in the last two rows may be considered to be the values of the osmotic pressure of the protein particles.

[6] Dakin, H. D., *J. Biol. Chem.*, 1920, xliv, 499.

A similar experiment was made with different concentrations of solutions of the chloride of crystalline egg albumin. The original pH of the albumin chloride solution was 3.5 and that of the outside solution 3.0. After equilibrium was established the pH both inside and outside was slightly changed as is shown in Table V. The osmotic pressures for $\frac{1}{4}$ to 4 per cent solutions of albumin chloride were measured and calculated for $2y + z - 2x$. The difference, which should be the osmotic pressure of the albumin particles in solution, is found in the last row. It is almost identical with the difference found for gelatin chloride for the same concentration of gelatin.

V. Difference between the Curves for Calculated and Observed Values.

The curves representing the values for calculated osmotic pressures differ in one or two respects from the curves representing the values for the observed osmotic pressures. These differences are not great but they are constant and can therefore not be due to an accidental error. Fig. 3 shows the difference between the curves for the observed and the calculated osmotic pressures in the case of a gelatin chloride solution containing 1 gm. of originally isoelectric gelatin in 100 cc. If we start with the ascending branch of the two curves of Fig. 3, we notice that the observed osmotic pressures for pH 4.4 to pH 3.8 are about 100 mm. higher in each case than the calculated pressures. It may be a mere accident but 100 mm. happens to be the approximate value for the osmotic pressure of 1 gm. of gelatin in 100 cc. and if the gelatin particles participate in the osmotic pressure of solutions of gelatin salts our calculated values should be about 100 mm. lower than the observed values for the same pH in the case of a 1 per cent gelatin solution. Near the summit the difference becomes a little less but we have seen that we may expect such irregularities on account of experimental error. Besides at pH 3.4 the concentration of the gelatin solution was diminished by about 20 per cent on account of diffusion of water into the gelatin bag. The difference in the ascending branch of the observed and calculated values occurs in every experiment.

The second constant difference between the curves for observed and calculated osmotic pressures lies in the fact that the drop in the calculated curves begins at a lower pH than the drop for the curves of

observed values. This or both discrepancies may be due to a constant experimental error or they may find their explanation in the influence of one or more factors not taken into consideration in our calculations.
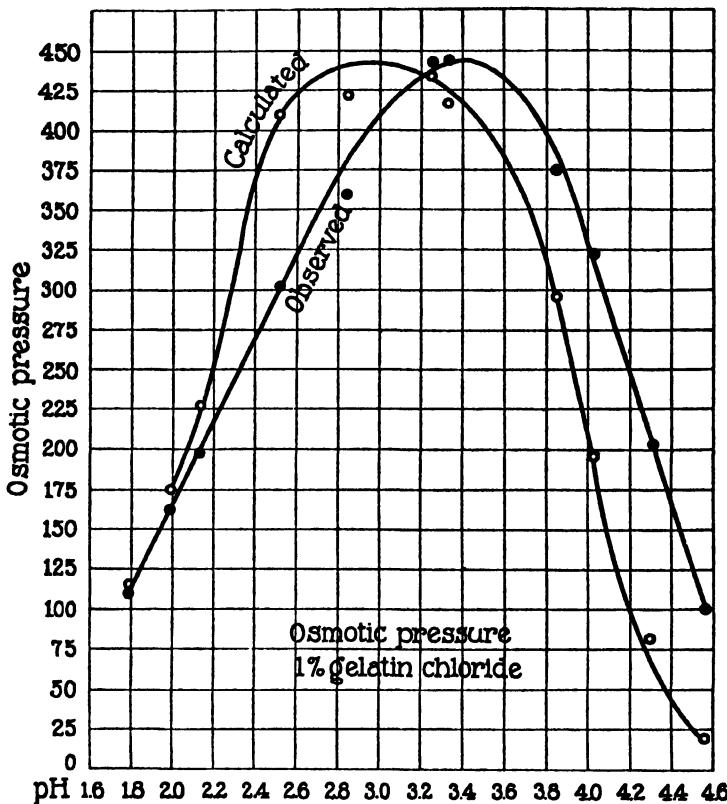
FIG. 3. Showing agreement and minor discrepancies between the curves of observed and calculated osmotic pressures of 1 per cent gelatin chloride solutions.

The calculated and observed curves for gelatin phosphate are identical with those for gelatin chloride given in Fig. 3, and we therefore omit these curves.

Fig. 4 gives the curves for observed and calculated values for gelatin sulfate. It seemed of interest to calculate the osmotic pres-

sures from some of our older experiments on the osmotic pressure of crystalline egg albumin (containing 1 gm. of isoelectric albumin in 100 cc. solution). Fig. 5 gives a comparison of the curves for the
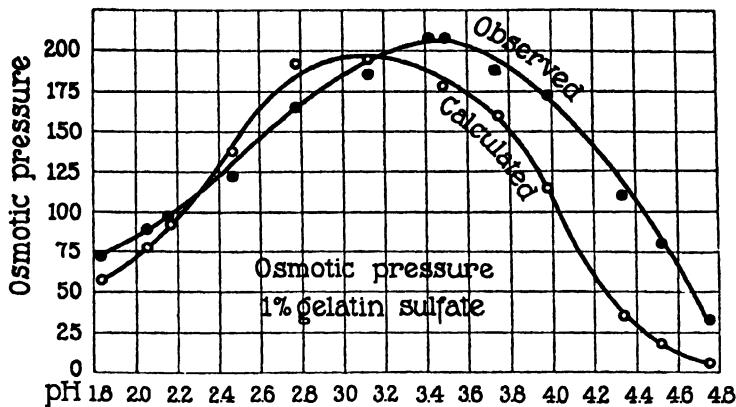
FIG. 4. Comparison of curves for observed and calculated values of osmotic pressure of solutions of 1 per cent gelatin sulfate.
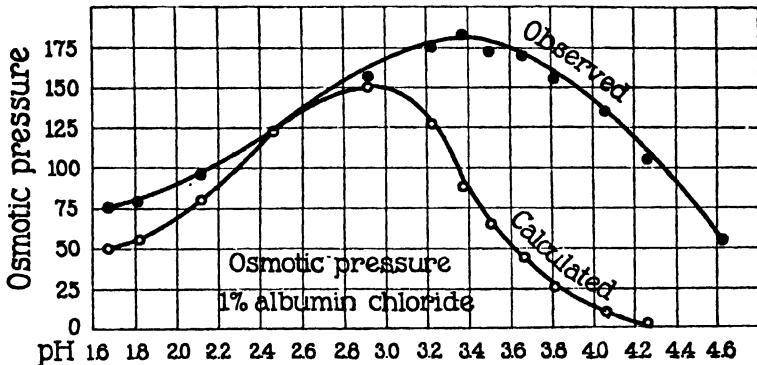
FIG. 5. Comparison of curves for observed and calculated values of osmotic pressure of solutions of 1 per cent crystalline egg albumin chloride.

observed and calculated osmotic pressures of 1 per cent albumin chloride and Fig. 6 the curves for observed and calculated osmotic pressures of 1 per cent albumin sulfate. In Fig. 6 appears the constant difference between the curves for observed and calculated

values which the neglect of the protein value in the calculated curves postulates.

Procter assumes in his theory of swelling that the protein particles do not participate in the osmotic pressure inside the gel, and the writer is willing to admit that the same assumption may be necessary for the osmotic pressure of the protein solutions. The fact that the maximal observed osmotic pressure of gelatin solutions agrees with the maximal pressure calculated, without regard to the possible osmotic pressure caused by the protein ions, seems to agree with Procter's view. In this case, we should have to say that the curves for the observed values coincide with the curves for the calculated values if the latter curves are moved to the right, parallel with themselves.
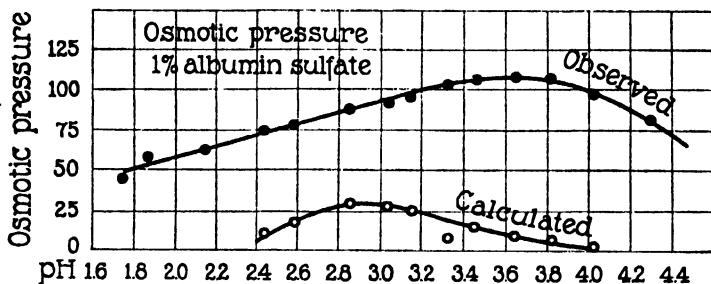
FIG. 6. Comparison of curves for observed and calculated values of osmotic pressure of solutions of 1 per cent crystalline egg albumin sulfate.

VI. The Depressing Influence of Neutral Salts on the Osmotic Pressure of a Gelatin Chloride Solution.

The question arises whether the membrane equilibrium could also be responsible for the depressing influence of salts on the osmotic pressure of protein solutions of a given pH and concentration of originally isoelectric protein. Although Donnan has shown that such a result is to be expected from his theory, the theory does not include all the facts in the case of gelatin chloride solutions. If on one side of the membrane there be a solution of NaCl, on the other side a colloidal salt NaR, where R is the colloidal ion, the real osmotic pressure (P_o) of the colloidal solution NaR can, according to Donnan,

be calculated from the observed osmotic pressure (P_1) by the following equation,

$$\frac{P_1}{P_o} = \frac{C_1 + C_2}{C_1 + 2C_2}$$

assuming that gelatin is monovalent, which is improbable, where C_1 is the concentration of NaCl and C_2 that of NaR. If C_1 is small compared with C_2, $P_1 = \frac{1}{2} P_o$; if, however, C_2 is small in comparison with C_1, $P_1 = P_o$. It follows from this that the greater the concentration of NaCl (or of any neutral salt) added to a colloidal solution, the smaller the observed osmotic pressure of the colloidal solution becomes, and this is what actually happens. It follows also from this theory that the observed osmotic pressure cannot be depressed below a certain minimum. This seems to agree with the observation that when the solution of the salt reaches the value M/8 a further increase in the concentration of the salt can no longer increase the depressing effect of the salt (see Figs. 1 and 2 in the preceding paper of this series).[2] To this extent Donnan's theory accounts for the depressing action of the salt on the osmotic pressure. A discrepancy between theory and observed value of osmotic pressure arises, however, in the fact that according to the theory the maximal depression caused by a salt should be $\frac{1}{2}$ the real osmotic pressure of the colloidal solution. The observed osmotic pressure of a 1 per cent solution of gelatin chloride of pH 3.5 is about 425 mm. H_2O while the osmotic pressure in the presence of M/8 NaCl is only about 30 mm. According to the theory the latter value should be at least $\frac{1}{2}$ of 425; i.e., 212.

Donnan's theory can only give approximate results in this case since in his theory complicating factors were intentionally ignored for the sake of simplification. Thus the depressing effect of the addition of a neutral salt on the electrolytic dissociation of the electrolyte is not taken into consideration.

Northrop[7] has shown by conductivity measurements that the degree of ionization of gelatin chloride is noticeably depressed when the pH is 2.0 or below and is considerable when the pH is 1.0. There may be other complicating factors besides this depression of ionization.

[7] Northrop, J. H., *J. Gen. Physiol.*, 1920–21, iii, 211.

VII. Theoretical Remarks.

It is of interest to compare the colloidal speculations with the almost quantitative results at which we arrived. All colloidal theories would agree in ascribing the effect of the hydrogen ion concentration or of the valency of the ions with which the protein is in combination to a modification in the state of the protein particles, such as hydratation or degree of dispersion. The hydratation theory
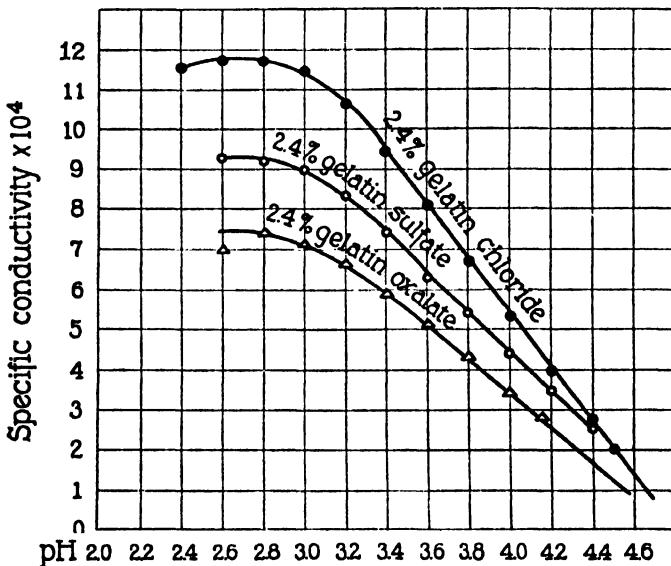
FIG. 7. Curves for the specific conductivity of 2.4 per cent solutions of gelatin chloride, sulfate, and oxalate, showing the entirely different character of these curves from that of the osmotic pressure curves in Figs. 1 and 5 in the preceding paper.

is advocated by Pauli[8] who assumes that the ionized protein particle is strongly hydrated while the non-ionized protein particle is not hydrated. If this were the correct explanation of the peculiar type of curves, such as those reported in Fig. 1 of this paper, we should expect the curves for the specific conductivity of the protein in solution to show a close similarity to the curves in Fig. 1. But such is

[8] Pauli, W., Kolloidchemie der Eiweisskörper, Dresden and Leipsic, 1920.

not the case. In Fig. 7 are given the curves for the specific conductivity of 2.4 per cent solutions of gelatin chloride, sulfate, and oxalate after deduction of the specific conductivity of the free acid in the gelatin solution, as described in a previous paper. A comparison between Fig. 7 and Fig. 1 fails to show any close similarity. In the conductivity curves there is no maximum followed by a drop at pH 3.5, as there is in the osmotic pressure curves. Fig. 8 shows that the
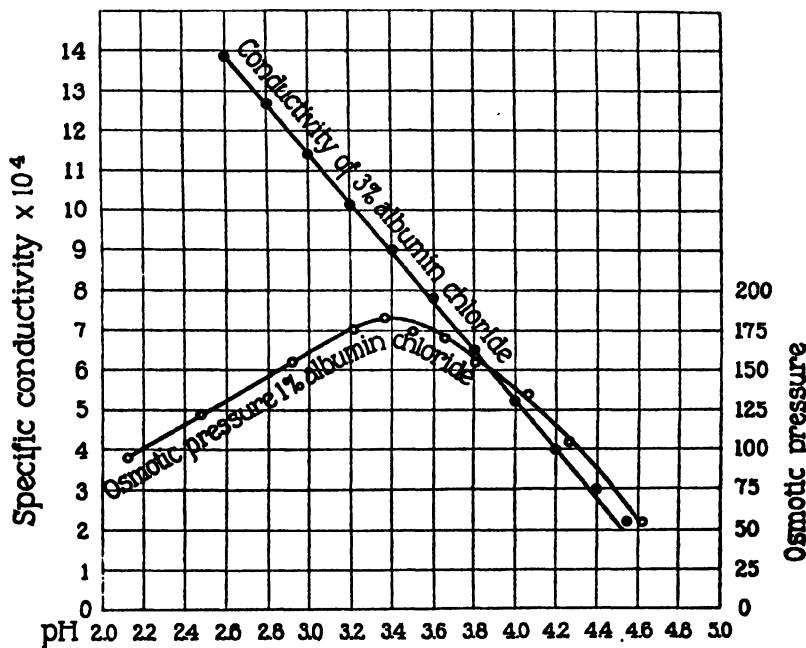
FIG. 8. Comparison of conductivity curve and osmotic pressure curve for albumin chloride, showing the entirely different character of the two curves.

difference between the conductivity curve and the osmotic pressure curve is still more pronounced in the case of albumin chloride.

Manabe and Matula,[9] who claim to have proved Pauli's theory, speak of a maximum of the physical properties of protein solutions at pH 2.0 or 2.1. There seems to be some misunderstanding, since the maximum for osmotic pressure of solutions of protein-acid salts;

[9] Manabe, K., and Matula, J., *Biochem. Z.*, 1913, lii, 369.

e.g., gelatin or crystalline egg albumin, lies at a pH of 3.5 or 3.4, and for casein at a pH above 3.0.   The maximal swelling lies at a pH of gelatin of about 3.2 or 3.3.   Those who state that it lies at a lower pH (*e.g.* Michaelis[10]) must have mistaken the pH of the supernatant liquid for the pH of the solid gelatin, thus ignoring the effects of the Donnan equilibrium.   The maximum for the viscosity of gelatin-acid salts lies at pH of 3.0 and for the viscosity of casein chloride or phosphate at pH 3.0 or above.

Northrop[7] has observed that a drop in the conductivity of gelatin solutions occurs when the pH falls below 2.0, but this cannot explain the drop in the osmotic pressure curves observed at pH above 3.0, ' and Northrop's results agree entirely with my own in not having noticed a drop in the conductivity curves at pH 3.0 or 3.5.

Moreover, Fig. 7 shows that there is only a slight difference between the conductivity curves for gelatin sulfate and gelatin chloride, while there is a greater difference between the conductivity of gelatin chloride and gelatin oxalate.   All this disagrees entirely with the osmotic pressure curves in Fig. 5 in the preceding paper.   Furthermore, the idea of a noticeable hydratation of the protein ion seems to be no longer tenable on the basis of Lorenz[11] and Born's[12] experiments and conclusions.

A second colloidal hypothesis would lead us to assume that variations in the degree of dispersion of the protein particles are responsible for the osmotic pressure curves represented in Fig. 1.   We need not dwell on this hypothesis since we have no way of putting it to a quantitative test.

The results of this paper show that if we assume the correctness of Donnan's theory of membrane equilibrium the characteristic influences of pH and valency on the osmotic pressure appear as a necessary consequence of the theory; with the exception of the two minor differences discussed in Chapter IV.   Donnan's theory leads to a view radically different from all colloidal speculations since on the basis of this theory the variations in osmotic pressure depend on the

[10] Michaelis, L., Praktikum der physikalischen Chemie insbesondere der Kolloidchemie, Berlin, 1921.

[11] Lorenz, R., *Z. Elektrochem.*, 1920, xxvi, 424.

[12] Born, M., *Z. Elektrochem.*, 1920, xxvi, 401.

unequal distribution of the crystalloidal ions on the opposite sides of the membrane and not on variations in so called colloidal properties of proteins. The quantity which changes with the pH and the valency of the anion of a protein-acid salt is on this assumption not the degree of hydratation or dispersion of the protein particles but the value pH inside minus pH outside, as stated more fully in the two preceding papers.[1]

SUMMARY.

1. It had been shown in previous publications that the osmotic pressure of a 1 per cent solution of a protein-acid salt varies in a characteristic way with the hydrogen ion concentration of the solution, the osmotic pressure having a minimum at the isoelectric point, rising steeply with a decrease in pH until a maximum is reached at pH of 3.4 or 3.5 (in the case of gelatin and crystalline egg albumin), this maximum being followed by a steep drop in the osmotic pressure with a further decrease in the pH of the gelatin or albumin solution. In this paper it is shown that (aside from two minor discrepancies) we can calculate this effect of the pH on the osmotic pressure of a protein-acid salt by assuming that the pH effect is due to that unequal distribution of crystalloidal ions (in particular free acid) on both sides of the membrane which Donnan's theory of membrane equilibrium demands.

2. It had been shown in preceding papers that only the valency but not the nature of the ion (aside from its valency) with which a protein is in combination has any effect upon the osmotic pressure of the solution of the protein; and that the osmotic pressure of a gelatin-acid salt with a monovalent anion (e.g. Cl, NO_3, acetate, H_2PO_4, HC_2O_4, etc.) is about twice or perhaps a trifle more than twice as high as the osmotic pressure of gelatin sulfate where the anion is bivalent; assuming that the pH and gelatin concentrations of all the solutions are the same.

It is shown in this paper that we can calculate with a fair degree of accuracy this valency effect on the assumption that it is due to the influence of the valency of the anion of a gelatin-acid salt on that relative distribution of the free acid on both sides of the membrane which Donnan's theory of membrane equilibrium demands.

3. The curves of the observed values of the osmotic pressure show two constant minor deviations from the curves of the calculated osmotic pressure. One of these deviations consists in the fact that the values of the ascending branch of the calculated curves are lower than the corresponding values in the curves for the observed osmotic pressure, and the other deviation consists in the fact that the drop in the curves of calculated values occurs at a lower pH than the drop in the curves of the observed values.

DONNAN EQUILIBRIUM AND THE PHYSICAL PROPERTIES OF PROTEINS.

III. VISCOSITY.

BY JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, May 20, 1921.)

I. Volume of Solute and Viscosity.

The viscosity of freshly prepared gelatin solutions is affected in a similar way by the pH as are the electromotive forces, the osmotic pressure, and the swelling. We have been able to show that the influence of the pH on the E.M.F. can be accounted for quantitatively on the basis of the Donnan equilibrium,[1] and that with the exception of one or two minor deviations the same is true for the osmotic pressure.[2] Procter and Wilson's theory of swelling is also based on Donnan's theory of membrane equilibrium.[3] Fig. 1 is the expression of the influence of the pH on the viscosity of 0.5, 1, and 2 per cent freshly prepared gelatin chloride solutions at a temperature of 24°C. The abscissæ are the pH of the gelatin solution, while the ordinates are the relative viscosities of the gelatin solutions compared with that of water at the temperature of the experiment.

These curves are modified if the viscosity of the gelatin solution is not measured immediately, but only after the solution has been standing for some time. In this case the curve changes inasmuch as the viscosity rises everywhere but the more rapidly, the nearer the pH is to that of the isoelectric point. In this case the curves representing the influence of pH on the viscosity of gelatin solution no longer resemble the curves representing the influence of the pH

[1] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 577, 667.

[2] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 691.

[3] Procter, H. R., *J. Chem. Soc.*, 1914, cv, 313. Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307.

on the osmotic pressure and swelling. In the literature, however, it is usually stated that the influence of acid on viscosity resembles that of acids on osmotic pressure and swelling, and the question arises whether or not the theory of the Donnan equilibrium can be applied to the explanation of this type of viscosity curves found when freshly prepared gelatin solutions are used (Fig. 1).
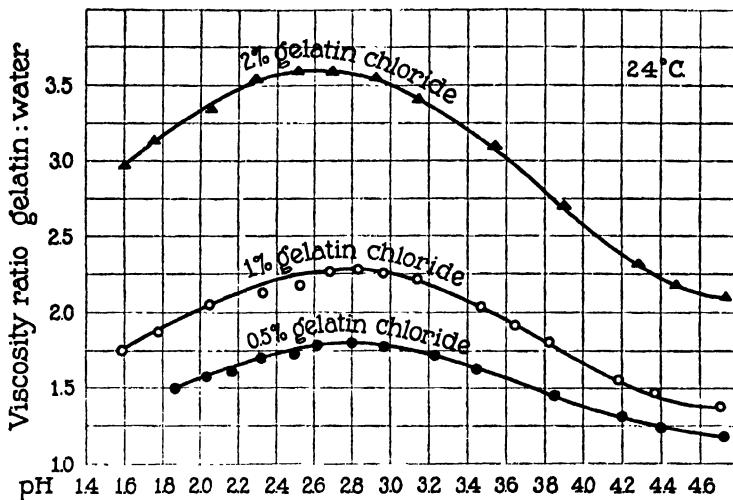
FIG. 1. Influence of pH on viscosity of freshly prepared gelatin chloride solutions.

Several formulæ exist for the calculation of the influence of a solute on the viscosity of a solvent. The first one (1) was derived by Einstein[4]

$$\eta = \eta_0 \, (1 + 2.5 \, \varphi) \qquad (1)$$

where η_0 is the viscosity of the pure solvent at the temperature of the experiment, η the viscosity of the solution, and φ the fraction of the volume occupied by the solute in the solution. This formula holds only when φ is very small and when the particles of the solute are spherical and large compared with the molecules of the solvent.

[4] Einstein, A., *Ann. Physik*, 1906, xix, 289; 1911, xxxiv, 591.

Hatschek,[5] Smoluchowski,[6] Hess,[7] and Arrhenius[8] have modified Einstein's formula so as to make it valid for any concentration. Arrhenius replaces the linear by a logarithmic formula

$$\text{Log } \eta - \text{Log } \eta_0 = \theta\varphi \qquad (2)$$

where φ is again the fraction of volume occupied by the solute in the solution and θ a constant, while η and η_0 have the same significance as in Einstein's formula.

All the formulæ agree in one point, namely that the fraction of the volume occupied by the solute in the solution is the main variable upon which the relative viscosity of a solution depends. It has been pointed out by Odén[9] and others that in addition to the relative volume occupied by suspended particles the average size of the individual granules in a suspension plays also a rôle in viscosity. According to these theories of viscosity, it should be possible to correlate the characteristic influence of the hydrogen ion concentration upon the viscosity of gelatin solutions with a variation in the relative volume or the average size of the gelatin particles in solution, since the mass of gelatin in solution remains the same in these experiments.

The measurement of the viscosity is in our experiments the time of outflow of the solution through a capillary tube and the method of the experiments (already described in a previous paper) was briefly as follows. To 50 cc. of a 2 per cent solution of isoelectric gelatin is added the desired acid, e.g., HCl, in sufficient quantity and then the volume is raised to 100 cc. by the addition of enough distilled water. This 1 per cent solution of originally isoelectric gelatin is rapidly heated to 45°C., kept at that temperature for 1 minute, and then rapidly cooled to 24°C. (or any other desired temperature). The viscosity is measured immediately after the solution was cooled to 24°C., since on standing the viscosity increases unequally at different pH. The measurements were all made by determining the time of outflow through a capillary tube. The time of

[5] Hatschek, E., *Kolloid Z.*, 1913, xii, 238; 1920, xxvii, 163.

[6] Smoluchowski, M. v., *Kolloid Z.*, 1916, xviii, 190.

[7] Hess, W. R., *Kolloid Z.*, 1920, xxvii, 1, 154.

[8] Arrhenius, S., *Meddelanden from K. Vetenskapsakademiens Nobelinstitut*, 1917, iii, No. 21.

[9] Odén, S., *Nova acta regiae Societatis Scientiarum Upsaliensis*, 1913, iii, No. 4.

outflow for pure water was 56 seconds at 24°C. The pH of the solutions was determined with the aid of the potentiometer.

The reader will notice (Fig. 1) that the relative viscosity of a gelatin solution is a minimum at the isoelectric point (pH = 4.7), that it rises with a rise in the hydrogen ion concentration until it becomes a maximum at pH of about 2.7, and that it drops again with a further rise in the hydrogen ion concentration. The question is how to explain the apparent changes in the relative volume of the gelatin in solution which, according to the theory, must be the main cause of the variation of the viscosity with the pH.

A change in the ratio of volume of gelatin to volume of water is only possible if water is added to the gelatin. Pauli[10] suggested that the ionized particle of protein is surrounded by a shell of water which is lacking in the non-ionized molecule. The volume of the protein ions in solution is increased by this jacket of water. Since the gelatin in solution is practically non-ionized at the isoelectric point, the relative volume of the gelatin in solution is a minimum at this point, while when we add an acid, e.g., HCl, gelatin chloride is formed, which, like all salts, ionizes readily. On this basis we can understand why the viscosity should increase with an increase of the hydrogen ion concentration of the gelatin solution; since with this increase in the hydrogen ion concentration, the concentration of hydrated gelatin ions and hence the volume of the gelatin particles should also increase. The work of Lorenz,[11] Born,[12] and others casts, however, a doubt on the assumption of a general hydration of polyatomic ions. We shall see presently that there are still other facts which show that the mere ionization and consequent hydration of the individual protein ions cannot well be the cause of the influence of the pH on the relative viscosity of gelatin solutions.

II. The Influence of the Hydrogen Ion Concentration on the Viscosity of Solutions of Amino-Acids and of Crystalline Egg Albumin.

If hydration of the individual protein ions were the cause of the variation of the viscosity of gelatin solutions, a variation of the hy-

[10] Pauli, W., Kolloid Z., 1913, xii, 222.

[11] Lorenz, R., Z. Elektrochem., 1920, xxvi, 424.

[12] Born, M., Z. Elektrochem., 1920, xxvi, 401.

drogen ion concentration should have a similar influence on the viscosity of solutions of simple amino-acids, like glycocoll and alanine, as it has on the viscosity of gelatin solution. 5 per cent solutions of glycocoll and alanine were brought to different pH, from 5.0 to 2.0 and below, by the addition of HCl. The variation of the pH of 5 per cent solutions of these two amino-acids between the limits of 5.0 and 1.16 had no measurable influence on the viscosity of the solution. This cast a serious doubt on the assumption that the variations in the curve of the viscosity of gelatin, as expressed in Fig. 1, were caused by variations in the hydration of the individual gelatin ions.[18]
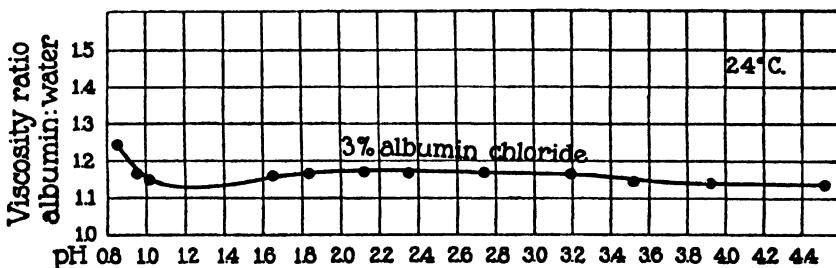
FIG. 2. Showing that solutions of crystalline egg albumin have a low viscosity in comparison with gelatin solutions, and that the pH has little influence on the viscosity of solutions of crystalline egg albumin at pH over 1.0 and at ordinary temperature.

This doubt was increased by experiments on the influence of pH on the viscosity of crystalline egg albumin which gave also a practically negative result. Fig. 2 gives such an experiment with 3 per cent originally isoelectric albumin brought to different pH through the addition of HCl. The ordinates are the viscosity ratios of albumin solution over water, drawn on a larger scale than those in Fig. 1, and the abscissæ are the pH of the solution. It is obvious that the pH has only a very slight if any influence on the viscosity of solutions of crystalline egg albumin between pH 4.6 and pH 1.0. With a further lowering of pH the viscosity suddenly rises, a fact to which we shall return later. It is also obvious that the viscosity

[18] These experiments were carried out by Dr. Elizabeth Brakeley.

ratio, protein solution: water, is considerably smaller in the case of albumin solutions than in the case of gelatin solutions.

The method of the experiments was as follows. 50 cc. of a 6 per cent solution of isoelectric crystalline egg albumin were mixed with 50 cc. of HCl solution of different concentration and the pH measured. The solution was rapidly brought to a temperature of 24°C. and the viscosity was measured immediately at that temperature.

The question then arises, why do amino-acids and at least one protein, namely crystalline egg albumin, behave so differently from gelatin in regard to the influence of the pH on the viscosity? As long as we assume that the influence of the hydrogen ion concentration on the viscosity of gelatin-acid salt solution is due to the hydration of the individual protein ions this difference is incomprehensible since the amino-acids as well as crystalline egg albumin should in this case show the same influence of ionization on hydration as the gelatin.

The puzzle becomes still greater if we take into consideration the fact that the osmotic pressure of solutions of crystalline egg albumin is affected in the same way by the hydrogen ion concentration as is the osmotic pressure of gelatin solutions. Why then do these two proteins behave so differently as regards the influence of the pH on their viscosity?

To answer this question we are forced to the conclusion that gelatin in solution must possess a way of increasing its volume which is lacking in the case of solutions of crystalline egg albumin (at least at ordinary temperature and at a pH above 1.0). This difference seems to be connected with a difference in the ability to form a gel. Solutions of isoelectric crystalline egg albumin of a high concentration can be kept for many months at a temperature just above the freezing point without setting to a jelly or without even showing an increase in viscosity; while solutions of isoelectric gelatin of even a low concentration show a rapid increase in viscosity and may set to a jelly under the same conditions of temperature and pH which do not alter the viscosity of egg albumin. Moreover, crystalline egg albumin has a very low viscosity compared with that of the same mass of gelatin in solution.

When, however, the pH of a 3 per cent solution of crystalline egg albumin falls to 0.85 or below, the solution can set to a gel and in that case its viscosity rises to the same order of magnitude as the viscosity of gelatin solutions except that an increase in temperature has the opposite effect as in the case of gelatin solutions. This is illustrated by Figs. 3 and 4. Both figures show the influence of time on the
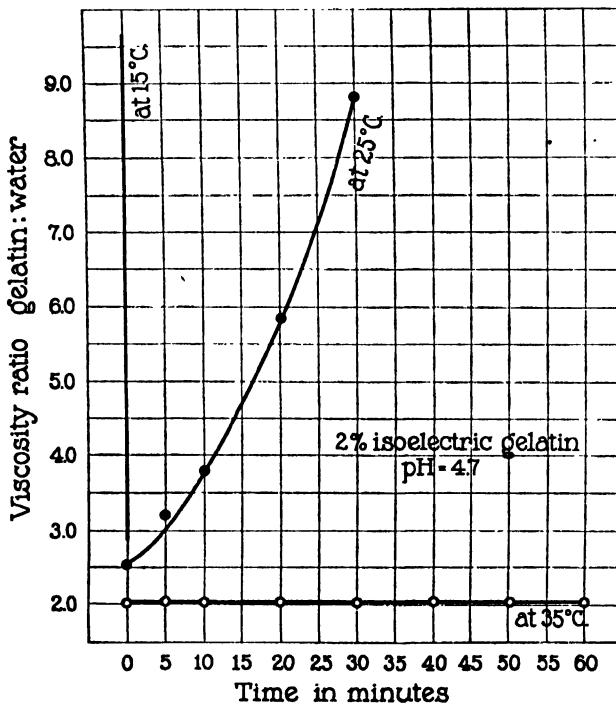
FIG. 3. Influence of time on viscosity of isoelectric gelatin solutions at different temperatures.

viscosity ratio of solutions of gelatin or albumin to that of pure water. Fig. 3 shows that the viscosity of a 2 per cent solution of isoelectric gelatin rises rapidly at 15°C., more slowly at 25°C., and quite slowly at 35°C.

Fig. 4 gives the influence of time on the viscosity of 3 per cent solutions of albumin chloride at pH 0.85 where the solutions are

opalescent and have a tendency to set to a gel. The reader will notice that at that pH viscosity of solutions of albumin chloride behaves like the viscosity of gelatin solutions, inasmuch as the viscosity of albumin chloride solutions also rises with time as soon as
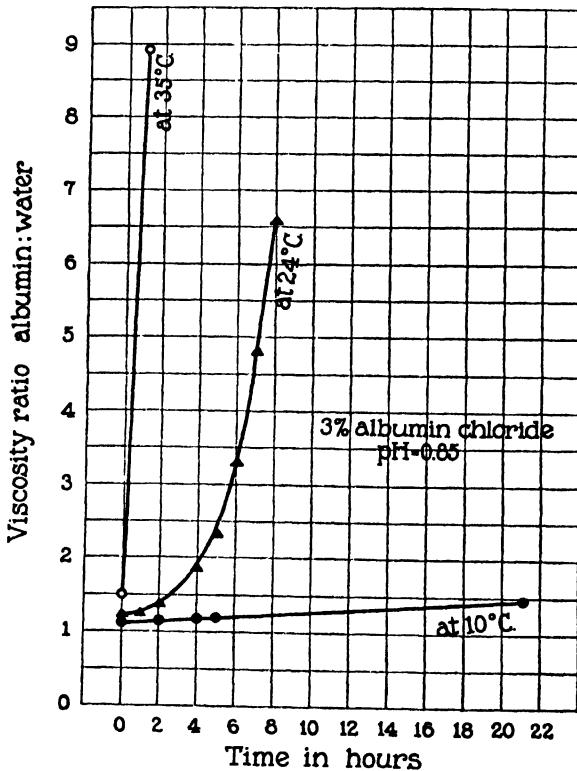
FIG. 4. Influence of time on viscosity of solutions of crystalline egg albumin of pH 0.85 at different temperatures. At this pH the albumin chloride solutions have a tendency to set to a jelly and have the same high order of viscosity as the gelatin solutions.

the solution acquires the property of setting to a gel, and that the order of magnitude of the viscosity of albumin chloride solutions capable of setting to a gel no longer differs from that of gelatin solution. The only difference is that of the influence of temperature on the viscosity which is the reverse in the case of the two proteins.

High temperature favors jelly formation in the case of egg albumin and retards it in the case of gelatin.

These observations corroborate the suspicion that the high order of magnitude of viscosity of gelatin solutions may be in some way connected with the tendency of this protein to set to a gel.

We do not yet know how the tendency of a protein solution to form a gel can account for the following two facts: first, that this tendency is accompanied by a rise in viscosity, and second, that the pH influences the viscosity in a way suggestive of the Donnan equilibrium. One possible answer to this question might be that the formation of a continuous gel by a protein solution may be preceded by the formation of a number of submicroscopic particles of gel, each occluding a considerable amount of water. This occlusion of water would cause a considerable increase in the volume of the mass of gelatin and this could account for the rise in viscosity with the tendency to form a gel. The Donnan equilibrium would regulate the quantity of water occluded by each particle and this would account for the influence of pH. The idea of such a possibility gave rise to the following experiments on the viscosity of suspensions of powdered gelatin in water.

III. Influence of Volume of Powdered Gelatin on Viscosity.

When we suspend finely powdered gelatin in water of a sufficiently low temperature, and measure the viscosity of such suspensions we find that they may have even a higher viscosity than gelatin solutions of the same concentration of gelatin and that the pH influences the viscosity of the suspension in the same characteristic way as that expressed in the curves of Fig. 1.

0.5 gm. of Cooper's powdered commercial gelatin of a pH of about 6.0 was added to 100 cc. of water containing varying amounts of HCl. The particles had uniform size (going through Sieve 100 but not through Sieve 120), but their shape was extremely irregular. They were left in the solution several hours at 20°C., and then their time of outflow through a capillary tube was ascertained at 20°C. The time of outflow of water through the viscometer at this temperature was 24 seconds. It was essential to stir the suspension thoroughly

before sucking it into the viscometer since the gelatin particles sink rapidly to the bottom of the dish.

After the viscosity measurements were taken, the suspension was put on a filter of cotton wool and the supernatant water allowed to drain off. By measuring the volume of the filtrate and deducting this from the original volume of the suspension (which was in all cases 100 cc.), the volume of the gelatin (with some error) was obtained. Then the gelatin was melted, and the pH of the melted mass of gelatin as well as of the filtrate was determined potentiometrically. Fig. 5 gives the result of such an experiment. The lower
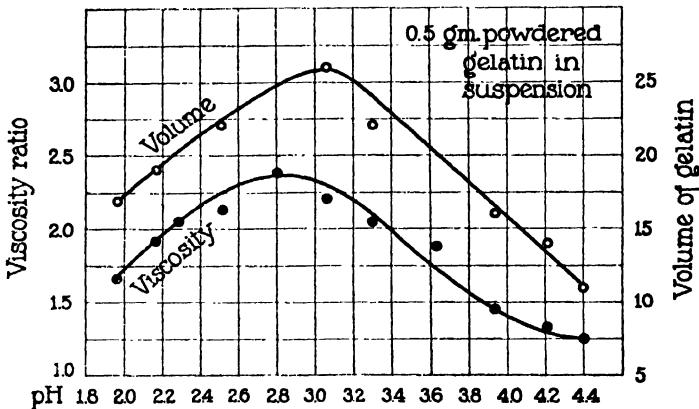
Fig. 5. Showing that the influence of pH on viscosity of 0.5 per cent suspensions of powdered gelatin in water is similar to the influence of pH on viscosity of gelatin solutions, and that the volume occupied by the particles in the suspension varies in a similar way as the viscosity. Temperature 20°C.

curve shows the influence of the pH (of the gelatin) on the viscosity, and the upper curve the influence of the pH on the volume of the gelatin. The two curves are similar.

Moreover, the viscosity values for the suspensions of 0.5 gm. of powdered particles in water are greater than the viscosity values of a 0.5 per cent solution of freshly liquefied gelatin for equal pH.[14] The highest viscosity ratio of the 0.5 per cent gelatin solution was about 1.75 while the highest viscosity ratio for the 0.5 per cent suspension of gelatin was about 2.4 (Fig. 5).

[14] The viscosity of the solution increases on standing.

These experiments prove, first, that a suspension of powdered gelatin in water shows the same variation in viscosity with the variation of the hydrogen ion concentration as does a solution of freshly prepared gelatin; and, second, that the relative volume of the suspended particles varies in a similar way as the viscosity (Fig. 5). In this case there is little doubt that the variations in the volume of the suspended particles of gelatin under the influence of the pH are due to the existence of a Donnan equilibrium between the particles and the surrounding water, since we have already shown in a former publication that there exists a difference in the pH of the solid particles of powdered gelatin and the supernatant water and this fact was further corroborated in these experiments (Table I).

TABLE I.

Donnan Equilibrium Between Suspended Particles of Gelatin and Supernatant Water After 20 Hours.

0.5 gm. of Gelatin Suspension in 100 cc. H_2O Containing Various Amounts of HCl at Temperature of 20°C.

pH of gelatin particles	4.79	4.62	4.33	4.17	3.93	3.60	3.26	3.02	2.68	2.39	2.16	2.07	1.80
pH of supernatant water	4.74	4.30	3.95	3.76	3.55	3.21	2.95	2.77	2.56	2.31	2.10	2.02	1.75

The point which is of importance is the question of the applicability of Einstein's formula to these experiments

$$\frac{\eta}{\eta_0} = 1 + 2.5\,\varphi$$

The fact that the shape of the suspended particles of gelatin is very irregular and that the average size of the individual particles plays also a rôle must warn us not to expect too strict an applicability of the formula in our case; and we may expect to obtain slightly different values than 2.5 for the constant. Since we can measure $\frac{\eta}{\eta_0}$ as well as φ directly in our experiments, we may write Einstein's equation in the form

$$\frac{\eta}{\eta_0} - 1 = c\varphi$$

and may try to calculate the value of $c = \dfrac{\dfrac{\eta}{\eta_0} - 1}{\varphi}$ from our observa-

tions. Since φ is the ratio of the volume of the gelatin to the volume of the solution and the latter is 100 cc., we have to multiply

the value $\dfrac{\dfrac{\eta}{\eta_0} - 1}{\text{volume of gelatin}}$ by 100 to obtain c.

Table II shows the results of such a calculation.

TABLE II.

pH of gelatin.	$\dfrac{\eta}{\eta_0}$	Volume of gelatin.	c
		cc.	
4.80	1.070	4.5	1.5
4.40	1.250	8.0	3.1
4.21	1.345	11.0	3.1
3.94	1.515	16.0	3.2
3.63	1.845	18.0	4.2
3.30	2.120	21.0	5.3
2.80	2.340	22.5	6.0
2.51	2.150	19.5	5.9
2.28	2.080	18.0	6.0
2.16	1.865	16.0	5.4
1.96	1.726	16.0	4.5

Considering the fact that the gelatin particles are not perfect spheres, as Einstein's theory demands, and considering the further fact that the measurements of the volume of gelatin are crude, it is surprising that where the volume of the gelatin is small the constant c is so near that expected on the basis of Einstein's formula, namely 3.1 instead of 2.5. Larger values are found (from 4.2 to 6.0) when the swelling of the gelatin particles becomes too large to permit the strict application of Einstein's formula. In all probability a second variable enters in this case, namely the large size of the individual granules. We shall see in a later paper that in the case of suspensions of gelatin particles the viscosity is not only a function of the relative volume of the suspended particles but also of their size, increasing (for the size used in our experiments) with the size. This might account for the fact that in Table II the value of c varies with the size of the particles.

If we consider all these complicating circumstances there can be little doubt left that the influence of pH on the viscosity of suspensions of particles of gelatin is mainly due to the change in volume of these particles under the influence of the pH and that this change of volume finds its explanation in the Donnan equilibrium between the particles and the surrounding liquid.

It is well known, and it has been discussed in preceding papers,[15] that the viscosity of a gelatin chloride solution, e.g., of pH 3.0, is
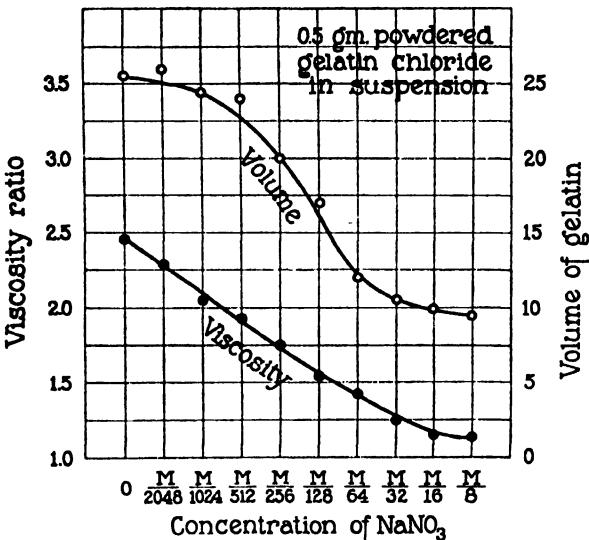
FIG. 6. Showing depressing influence of neutral salts on viscosity of suspensions of powdered gelatin in water and on the volume occupied by the gelatin particles in the suspension.

lowered when neutral salts are added and the pH kept constant. The same is true for the viscosity of suspensions of powdered gelatin. Doses of 0.5 gm. of powdered gelatin of pH 6.0, going through Sieve 100 but not through Sieve 120, were put each into 100 cc. of water containing 6 cc. of 0.1 N HCl, and different quantities of NaNO₃, so that the concentration of the salt varied in the different solutions from M/8 to M/2048. One solution contained no salt. The pH of the

[15] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 391.

gelatin varied in the neighborhood of 3.0; the temperature was 20°C. After 2½ hours, when the Donnan equilibrium between the particles and the surrounding solution was supposed to be established, the viscosity of each suspension was measured at 20°C. and the volume occupied by the suspended particles of gelatin was ascertained in the manner described. It was found that the addition of salt diminished the relative volume of the gelatin particles and the viscosity in a similar way (Fig. 6). The observed volume of the solutions containing little or no salt was probably a little too great on account of incomplete filtration.

The measurement of the pH of the gelatin solution and the outside ⸱ solution showed that the addition of salt diminished the difference between the two, as Donnan's theory demands (Table III).

TABLE III.

	Concentration of NaNO₃.								
	0	$M/2,048$	$M/1,024$	$M/512$	$M/256$	$M/128$	$M/64$	$M/32$	$M/16$
pH of gelatin particles.................	3.04	3.04	3.03	3.02	3.00	3.02	2.97	2.94	2.85
pH of supernatant liquid...............	2.74	2.76	2.76	2.76	2.77	2.80	2.78	2.77	2.70
Difference, pH inside minus pH outside...	0.30	0.28	0.27	0.26	0.23	0.22	0.19	0.17	0.15

SUMMARY AND CONCLUSION.

1. Gelatin solutions have a high viscosity which in the case of freshly prepared solutions varies under the influence of the hydrogen ion concentration in a similar way as the swelling, the osmotic pressure, and the electromotive forces. Solutions of crystalline egg albumin have under the same conditions a comparatively low viscosity which is practically independent of the pH (above 1.0). This difference in the viscosities of solutions of the two proteins seems to be connected with the fact that solutions of gelatin have a tendency to set to a jelly while solutions of crystalline egg albumin show no such tendency at low temperature and pH above 1.0.

2. The formulæ for viscosity demand that the difference in the order of magnitude of the viscosity of the two proteins should cor-

respond to a difference in the relative volume occupied by equal masses of the two proteins in the same volume of solution. It is generally assumed that these variations of volume of dissolved proteins are due to the hydration of the isolated protein ions, but if this view were correct the influence of pH on viscosity should be the same in the case of solutions of gelatin, of amino-acids, and of crystalline egg albumin, which, however, is not true.

3. Suspensions of powdered gelatin in water were prepared and it was found, first, that the viscosity of these suspensions is a little higher than that of gelatin solutions of the same concentration, second, that the pH influences the viscosity of these suspensions similarly as the viscosity of freshly prepared gelatin solutions, and third, that the volume occupied by the gelatin in the suspension varies similarly as the viscosity which agrees with the theories of viscosity. It is shown that this influence of the pH on the volume occupied by the gelatin granules in suspension is due to the existence of a Donnan equilibrium between the granules and the surrounding solution.

THE RÔLE OF THE ACTIVITY COEFFICIENT OF THE HYDROGEN ION IN THE HYDROLYSIS OF GELATIN.

BY JOHN H. NORTHROP.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, May 9, 1921.)

I. *The Course of the Reaction.*

It is generally assumed that enzymes, and catalysts in general, merely accelerate the velocity of a reaction already taking place. In the case of most enzymes the reaction without the enzyme has been carefully studied and is at least as well known as the enzyme reaction. The hydrolysis of proteins without the presence of enzymes, however, has apparently received very little attention, at least from the standpoint of the kinetics of the reaction. It seemed advisable, therefore, in connection with the study of the proteolytic enzymes to secure some data in regard to the spontaneous hydrolysis, especially since the writer had found that the hydrolysis of gelatin by pepsin[1] was very closely connected with the ionization of the protein. It seemed important to determine whether any influence of the ionization could be noticed in the spontaneous reaction. As will be described more fully below, this is found to be the case.

EXPERIMENTAL.

Preparation of the Gelatin.—Gelatin was selected as the protein in these experiments since it had already been used in the study of pepsin hydrolysis and also since it does not precipitate in strong acid and alkali. It is also much more accurately titratable by the formol titration than are the other proteins. The gelatin was prepared from Cooper's powdered gelatin by washing at the isoelectric point as described by Loeb,[2] and was used in a concentration of

[1] Northrop, J. H., *J. Gen. Physiol.*, 1920–21, iii, 211.

[2] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 237.

from 2 to 3 per cent.   It was practically salt-free.   Several different preparations were made during the course of the work.   No difference could be detected in the behavior of these different lots.

The increase in free amino or free carboxyl groups is the most significant quantity as regards the hydrolysis of proteins, since if the accepted views of structure of the proteins are correct, each hydrolytic cleavage results in the liberation of a free amino and a free carboxyl group.   Two methods are available for following quantitatively the course of such reaction; Van Slyke's[3] amino nitrogen determination, and Sörensen's[4] formol titration which determines the free carboxyl groups.   For absolute determinations of the amino-acids Van Slyke's method is more accurate, for comparative experiments concerning the changes occurring in gelatin solutions, such as were used in this work, the formol titration is more accurate and also much more rapid.   Such a slight hydrolysis of gelatin as that required merely to liquefy the protein may be quite accurately determined by the formol titration whereas the increase in amino nitrogen is so small as to be within the limits of error of the Van Slyke method. The formol titration was used therefore in nearly all the experiments reported in this paper.   It is well known that many substances, such as phosphates and carbonates, interfere with this titration. In order to avoid these difficulties the method was slightly modified. It is obvious that the final titration figure will depend on the amount of alkali or acid present in the original solution.   In order to get comparable results, therefore, it is always necessary to start the titration from the same pH.   This was accomplished by titrating the sample to pH 7.0, using neutral red as an indicator, before adding the formaldehyde and continuing with the final titration.   In the case of gelatin itself, this method does not give the correct figure since, in order to get the total acidity, it is necessary to start the titration at the isoelectric point of the gelatin; *i.e.*, pH 4.7.   The figures obtained by titrating from pH 7.0 are, therefore, too low by the amount of alkali necessary to titrate the gelatin from pH 4.7 to pH 7.0.   Since this is a constant quantity for any given concentration of gelatin, the titration figure could be corrected if necessary.

[3] Van Slyke, D. D., *J. Biol. Chem.*, 1913–14, xvi, 121.
[4] Sörensen, S. P. L., *Biochem. Z.*, 1908, vii, 45.

This correction is small, however, and in the case of comparative experiments cancels out. In the titration of the amino-acids the correction is within the limits of error of the titration. A pH of 7.0 was chosen as the standard since the titration curve of gelatin is very flat at this point so that the adjustment of the reaction is accurate to less than 0.05 cc. of 0.1 N NaOH and also since the presence of citrate does not interfere at this pH.

Adjustment of the pH of Gelatin Solutions.—In strongly acid or alkaline solutions it is not necessary to use buffers since the change in the pH during the hydrolysis of the gelatin is neglible. Between pH 1.5 and 11.0, however, it is necessary to use some buffer as otherwise the pH of the solution changes rapidly during the course of the reaction. It was noted above that phosphates and carbonates interfere with the titration. Phosphates are difficult to remove and were not used at all in these experiments. It was found that a complete series of buffer solutions could be prepared with various mixtures of trisodium citrate, HCl, and Na_2CO_3. These solutions were therefore used. Since carbonate also interferes with the titration it was removed by bringing the sample to about pH 2.5 with strong acid, boiling out the CO_2 and then titrating back to pH 7.0. Control experiments showed that this procedure had no effect whatever on the final titration; that is, the figure obtained for 10 cc. of gelatin solution was identical when the gelatin was titrated to pH 7.0 directly before making the formol titration, and when it was titrated first to say pH 10 with Na_2CO_3, then made acid, the carbonate boiled out, and then titrated to pH 7.0. The total concentration of citrate was not above 0.05 N, since more concentrated solutions caused a slight increase in the titration figure and rendered the adjustment to pH 7.0 more difficult.

Formalin Solution.—A solution of formalin was used containing 30 cc. of a saturated aqueous solution of thymol blue per 300 cc. of solution, and titrated with sufficient alkali, so that when 5 cc. were added to 15 cc. of water, the resulting solution had a pH of 8.4. This procedure obviates the necessity of making a correction for the formalin solution. 5 cc. of this solution were used for 10 cc. of the sample.

Example of Titration.—(*a*) *Solution Containing no Carbonate.*—10 cc. of 2.5 per cent gelatin solution or the equivalent amount, if more concentrated gelatin is used, are pipetted into a large test-tube, 1 drop of neutral red is added, and the solution is brought to approximately pH 7.0 by the addition of a few drops of concentrated alkali (carbonate-free) or strong HCl. The reaction is then corrected accurately to pH 7.0 with 0.1 N NaOH (CO_2-free) or 0.1 N HCl, using a standard of pH 7.0 in the comparator block. 5 cc. of the formalin solution are then added and the solution is titrated with 0.1 N NaOH to pH 8.4, using a standard tube with thymol blue for comparison. The neutral red does not interfere with this titration and if anything makes it more sharp. The number of cc. of 0.1 N alkali required to titrate from pH 7.0 to pH 8.4 after the addition of the formalin is noted and is referred to as the formol titration. This value, as Sörensen showed, is independent of the final pH taken, provided this is above 8.2. It represents the number of cc. of 0.1 N acid present in the solution.

(*b*) *Carbonate Present.*—The solution is brought to pH 2.5–3.0 with a few drops of concentrated HCl and boiled for 10 to 20 seconds over the free flame to remove the CO_2. It is then titrated as described under (*a*).

In order to check the accuracy of the method several experiments were made in which the results obtained by the formol titration and by Van Slyke's method were compared. The results of a few of these are given in Table I. The amino nitrogen was determined as described by Van Slyke. The solution was shaken for 30 minutes. In the case of gelatin, the titration figures for gelatin alone, without the addition of formalin are also given. These figures were obtained from the titration curves of gelatin given by Loeb,[5] by correcting for the amount of acid or alkali necessary to bring the same volume of water to the same pH. It is found that below pH 1.8 and above pH 11.0 at 25°C. the figures so obtained are constant and independent of the pH; *i.e.*, outside these limits, the gelatin is all combined as a salt and the figures represent the normality of the solution.

[5] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 85.

TABLE I.

Analysis of Gelatin Solutions by Different Methods.

Solution analyzed.	NH$_2$N per 2 cc. of solution, Van Slyke method.	Formol titration per 10 cc. of solution.	Equivalent cc. of 0.1 N NaOH calculated to 1 cc. 1 per cent gelatin.		Normality 1 per cent gelatin.
			Van Slyke.	Formol.	
	cc.	*cc.*	*cc.*	*cc.*	
5 per cent gelatin.	0.94	3.00 (direct ti-	0.036	0.060	0.0036 (Van
	0.88	3.05 tration.)	0.038	0.061	0.0038 Slyke).
		2.05 (titration to		0.040	0.0060 (Formol).
		pH 7.0.			
		2.03 first; neu-			
		tral red			
		titration.)		0.041	
5 per cent gelatin slightly hydrolyzed (just liquefied).	1.00	2.30 "	0.040	0.046	
	1.05	2.25 "		0.045	
2 per cent gelatin partially hydro-lyzed.	5.00	11.60 "	0.60	0.58	
	4.80	11.50 "	0.57	0.575	
2.5 per cent gelatin nearly completely hydrolyzed at pII 14.0 containing 0.1 N Na$_2$CO$_3$.	10.0	19.5 "	0.80	0.83	
	10.4	20.8 "	0.84	0.78	
1 per cent gelatin ti-trated directly with HCl or any strong acid.			0.120		0.012
1 per cent gelatin ti-trated directly with NaOH or Ba(OH).			0.06		0.006

The table shows that the formol titration, if the gelatin is first brought to pH 7.0, gives identical figures with that of the Van Slyke determination at all stages of hydrolysis. It also shows that the direct titration of gelatin without any formalin gives the same value

as that obtained with the formol titration. The formaldehyde evidently merely makes the acid groups stronger so that it is not necessary to titrate to such a high pH.

The figures in the last column show that 1 per cent gelatin solution is about 0.004 normal with respect to free NH_2 groups, 0.006 normal with respect to total acid groups, and 0.012 normal with respect to total basic groups. That is, there must be approximately twice as many basic as carboxyl groups, and three times as many total basic groups as NH_2 groups. These figures agree approximately with those found by Wintgen and Krüger.[6]

Determination of Hydrogen Ion Concentration.—The pH of the solutions was determined before and after hydrolysis. In the case of the buffered solutions no significant change was noted. The determination was made by the E.M.F. method (except in the solutions containing $HgCl_2$) using the rocking electrodes as described by Clark[7] in a constant temperature bath. A saturated KCl calomel electrode was used. The E.M.F. of this electrode was determined before and after every series of determinations against a 1.0 N solution of hydrochloric acid, prepared according to Hulett[8] and checked by conductivity measurements. The pH of this 1.0 N HCl was calculated from the activity coefficient as given by Noyes and MacInnes;[9] *i. e.*, 0.082 at 25°C. This standard was chosen since Fales and Vosburgh[10] have shown that there is no diffusion potential in such a cell and since the strength of the acid can be checked by an independent method; *i.e.*, the conductivity. The value for the saturated KCl electrode found in this way averaged 244 millivolts. It varied about 1 millivolt on either side of this value, from time to time, but was constant within 1 millivolt during the course of any one series of determinations.

Influence of Temperature on the pH.—Since the experiments were carried out at 40°C. and most of the pH determinations were made at 25°C. it was necessary to know what effect this change of tempera-

[6] Wintgen, R., and Krüger, K., *Koll. Z.*, 1921, xxviii, 81.

[7] Clark, W. M., Determination of hydrogen ions, Baltimore, 1920.

[8] Hulett, G. A., and Bonner, W. D., *J. Am. Chem. Soc.*, 1909, xxxi, 390.

[9] Noyes, A. A., and MacInnes, P. A., *J. Am. Chem. Soc.*, 1920, xlii, 239.

[10] Fales, H. A., and Vosburgh, W. C., *J. Am. Chem. Soc.*, 1918, xl, 1291.

ture would have on the pH. In order to determine the value of this temperature effect a series of gelatin solutions containing 2 per cent gelatin and having a pH of from 0.0 to 14.0 was made up. The pH was adjusted by varying amounts of HCl, NaOH, Na_2CO_3, and sodium citrate. The total salt content of all the solutions was between 0.02 and 0.05 N. The pH of these solutions was then determined at 25° and at 37°C. (The electrodes regulated at 37°C. were put at the author's disposal by Dr. Glenn E. Cullen). The results are summarized in Table II. The C_{OH} was calculated by the formula $C^H C_{OH} = K_W$ (or pH + pOH = Log K_W). The value of K_W at 25° was taken as 1×10^{-14} and at 40° as 2.8×10^{-14}. It was found that the hydrogen ion concentration was independent of the tem-

TABLE II.

Effect of Temperature on pH of Gelatin Solution.

pH observed.		pOH calculated.	
25°C.	40°C.	25°C.	40°C.
12.70	12.25	1.30	1.25
12.25	11.75	1.75	1.75
9.92	9.72	4.08	3.78
8.42	8.42	5.58	5.08
5.01	5.02	8.99	8.48
0.76	0.75	13.24	12.75

perature from pH 0.0 to pH 8.8, and that the (calculated) hydroxyl ion concentration was independent of the temperature from pH 11 to pH 14. Between pH 8.8 and 11.0 the concentrations of both hydrogen and hydroxyl ions increased with the temperature. In all the other experiments therefore the hydrogen ion concentration was assumed to remain the same at 25° and 40°C. on the acid side of pH 8.5, and the hydroxyl ion concentration was assumed to remain constant on the alkaline side of pH 11.0. The experiments which fell between pH 8.8 and 11.0 were measured at 37°C.

The Course of the Reaction.—It has been known for a long time that proteins may be hydrolyzed to the amino-acids by prolonged heating with acids or alkali. The kinetics of the reaction, however, have received little or no attention. Since it was desired to compare the

velocity of hydrolysis of the gelatin under different conditions it was first necessary to determine some value to be used as a standard. Two gelatin solutions were therefore prepared, one containing 0.75 N NaOH and the other 4.5 N HCl. These solutions were then placed at 65°C. and the increase in the formol titration followed. The results are shown in Fig. 1. The curves fall off more rapidly than the predicted rate of a monomolecular reaction, as is shown by Fig. 2, in which the logarithm of the quantity of gelatin remaining is plotted against the time, which in the case of a monomolecular
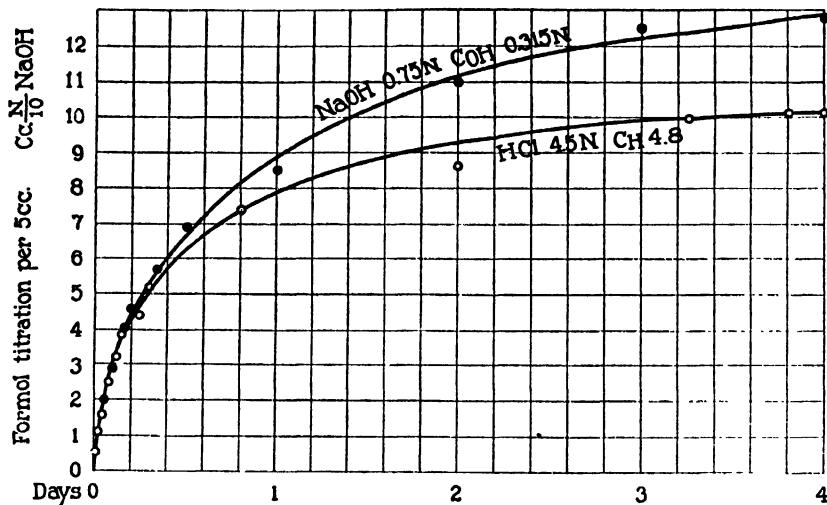
FIG. 1. Increase, with time, in formol titration in strongly alkaline and strongly acid solutions.

reaction gives a straight line. It is obvious from the figure that the reaction is monomolecular for the first 30 or 40 per cent but than becomes too slow. This cannot be ascribed to changes in the C_H or C_{OH} since no measureable change in these values could be detected. This means presumably that the simpler decomposition products are more resistant to hydrolysis than is the gelatin itself. Schroeder[11]

[11] von Schroeder, P., Z. physik. Chem., 1903, xlv, 75. Taylor (Taylor, A. E., Univ. California Pub., Path., 1907, i, 239) states that the hydrolysis of protamine by acid is monomolecular.

found that the rate of decrease of the viscosity of gelatin solutions also follows the monomolecular formula. This would be expected since the decomposition products have a very low viscosity compared to gelatin. It may be mentioned that the final figure reached by the two solutions was nearly identical and agreed with that found by Van Slyke for the total hydrolysis of gelatin. The alkaline solution gave a slightly higher figure due to silicic acid from the glass. Since the gelatin must evidently be destroyed in the very beginning of the reaction, it is this part which must be studied in order to notice any influence of the condition of the gelatin on the velocity of hydrolysis. It will be noticed that the formol titration increases about
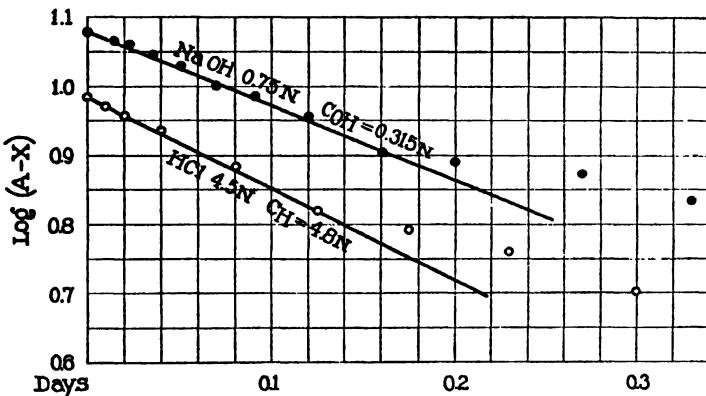
Fig. 2. Increase, with time, in formol titration in strongly aklaline and strongly acid solutions.

20 times during the reaction so that during the first two or three hundred per cent increase in the original figure the increase will be very nearly linear with respect to time.

That this is actually the case is shown in Fig. 3 in which the increase in the formol titration, as expressed in per cent of the original figure is plotted against the time. As the figure shows, the curves of all the experiments in which the hydrogen ion concentration was kept constant are straight lines within the limits of experimental error. Curve 4 in which the hydrogen ion concentration decreased during the hydrolysis, however, drops off quite rapidly. As will be shown

later, the velocity in this range of pH is proportional to the hydrogen ion concentration. Also since the products formed are weak bases we have the condition that[12]

$$RNH_2 + HCl \rightleftharpoons R\,NH_2.HCl \qquad (1)$$

and hence, when RNH_2 is large compared to C_H; (assuming complete ionization of the HCl)

$$C_H = \frac{k}{C_{RNH_2}}$$

i.e., the hydrogen ion concentration and hence the velocity of the reaction will be inversely proportional to the amount of products' formed.

$$\text{Velocity} = \frac{dx}{dt} = k\,C_H = \frac{k'}{C_{RNH_2}} = \frac{k'}{x}$$

This, as Arrhenius[13] pointed out is the condition which causes Schütz's rule since on integration it becomes

$$x = k''\,\sqrt{T}$$

In Fig. 4 the results have been plotted against the square root of the time and it will be seen that the experiment in which the hydrogen ion concentration was *not* kept constant (by means of buffers) obeys Schütz's rule very well; *i.e.*, the amount of products formed is proportional to the square root of the time. Reactions in which the rate is inversely proportional to the amount of products formed have been quite frequently observed. A very clear case which was completely worked out and the mechanism verified experimentally is that of the oxidation of oxalic acid by bromine studied by Richards and Stull.[14] Similar reactions are discussed by Müller.[15] The writer

[12] The complete equation would be

$$RNH_2 \begin{matrix} + HCl \rightleftharpoons RNH_2 \\ \\ + H_2O \rightleftharpoons RNH_3OH \end{matrix} \quad .\ HCl \rightleftharpoons \begin{matrix} RNH_3^+ + Cl^- \\ \updownarrow \\ RNH_2^- + H^+ \end{matrix}$$
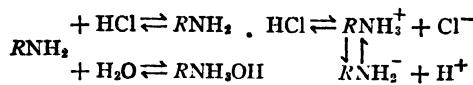
At the range of pH under consideration, however, the equilibrium may be represented approximately by (1).

[13] Arrhenius, S., *Medd. Kong. vetsakad. Nobelinst.*, 1908, i.

[14] Richards, T. W., and Stull, W. N., *Z. physik. Chem.*, 1902, xli, 544.

[15] Müller, W., *Z. physik. Chem.*, 1902, xli, 483.

was able to show[16] that in pepsin digestion the same mechanism causes the reaction to follow the square root law since the pepsin and the products of digestion are in equilibrium. The pepsin in this case replaces the hydrogen ion in the present example.

Returning to the experiments in which the hydrogen ion concentration was kept constant it will be seen that the rate of reaction is practically constant for at least the first 400 per cent increase in the original titration (equivalent to the first 20 per cent of the reac-
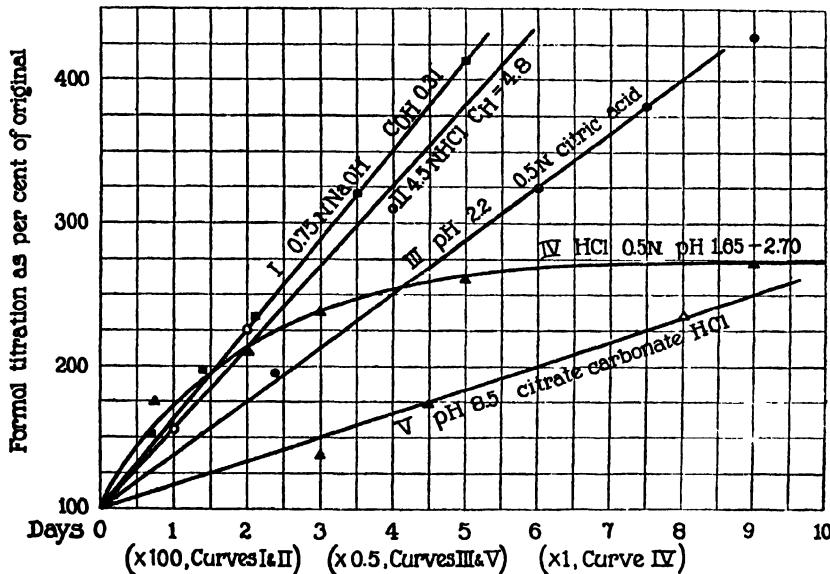
Fig. 3. Course of reaction with various hydrogen ion concentrations.

tion). The velocity within this range may, therefore, be determined at any time by dividing the increase in the titration by the elapsed time. If the increase is expressed as per cent of the original figure this ratio will represent the velocity of the reaction and will be independent of the concentration of gelatin. It seems more convenient and equally significant for the purposes of this paper to use this figure rather than the constant for the monomolecular reac-

---

[16] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 471.

tion. The velocity will therefore be considered as the increase expressed as per cent of the original titration divided by the elapsed time in days. The resulting figure is the per cent increase in the original titration per day, assuming that the amount of unhydrolyzed gelatin remains approximately unchanged. In most of the experiments determinations were made at several different times and the velocities so obtained were averaged. In all cases the experiment
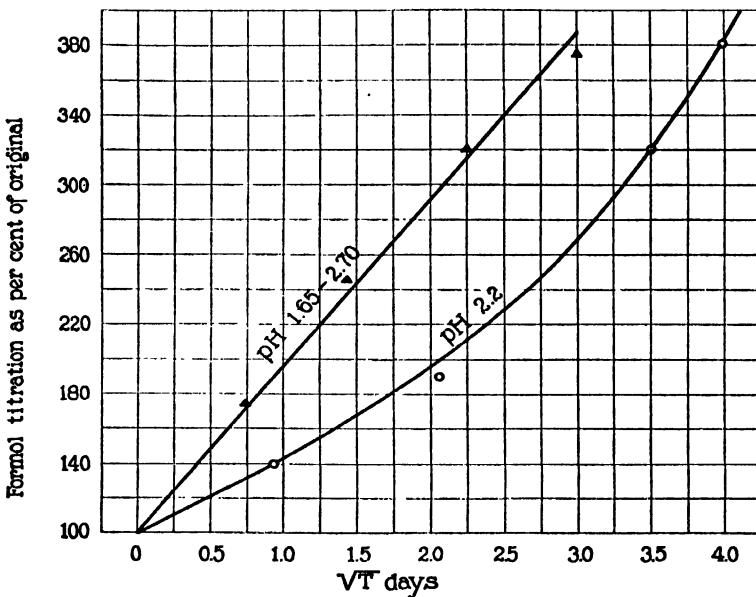
FIG. 4. Comparison of the course of reaction with constant and with varying hydrogen ion concentrations.

was continued until the original titration had increased at least 50 per cent and in all, except a few between pH 5.0 and 7.0, until the titration value had increased over 200 per cent.

Concentration of Gelatin.—Since the reaction approximates that of a monomolecular, the velocity when expressed as percentage change should be independent of the gelatin concentration. Table III contains the result of an experiment in which the gelatin concentration was varied from 10 to 1.25 per cent; it shows that the velocity is independent of the concentration for the early stages of the hydrolysis.

TABLE III.

Influence of Concentration of Gelatin at 65°C.
Gelatin Per Cent Concentration Noted + HCl to pH 2.0.

Concentration.	C_H	Velocity $= \dfrac{\text{per cent change}}{\text{time in days}}$.
per cent		
10	2.00	16
5	2.08	16, 14
2.5	2.03	17
1.25	2.02	17, 18

II. *The Influence of High Concentrations of Hydrogen and Hydroxyl Ions.*

It was shown above that the rate of the reaction decreased very rapidly if the hydrogen ion concentration was allowed to decrease. This in itself indicates that the rate is a function of the hydrogen ion concentration and not of the total amount of acid present. The fact that it is the hydrogen ion concentration and not the total acidity

TABLE IV.

Influence of Different Acids on Velocity of Hydrolysis.
2.5 Per Cent Gelatin at 65°C. with pH Measured at 25°C.

Acid.	Concentration.	C_H 25° $\times$ 10^3.	Velocity.	$\dfrac{\text{Velocity}}{C_H} \times 10^{-3}$.
	N			
HCl......................	0.024	6.0	18	3.0
HNO$_3$....................	0.024	6.0	17	2.8
H$_2$SO$_4$....................	0.024	6.3	16	2.5
Oxalic....................	0.050	6.3	16	2.5
Citric....................	0.33	5.6	16	2.8
		5.1	15	2.9

is shown more clearly in Table IV which contains the results of an experiment in which the gelatin was brought to the same pH with different acids. The table shows that the velocity is the same in all the solutions although the total concentration of citric acid was more than 10 times that of the HCl.

In all the above experiments the hydrogen ion concentration has been expressed as that calculated by Nernst's formula from the potential of the hydrogen electrode. It is certain that this value does not really express the actual concentration in grams per liter of hydrogen ions. It would probably be better to call it the "activity coefficient" or "active concentration" as proposed by Lewis;[17] that is, it represents that value which must be assigned to the hydrogen ion concentration in order to have it obey the law of mass action as assumed in Nernst's equation. In low concentration, however, the value so obtained does not differ very much from that obtained by the conductivity method. In more concentrated solution the discrepancy becomes greater and may amount to several hundred per cent in concentrated HCl. It seemed of interest to determine which of these values is the significant one for the hydrolysis of gelatin. A series of experiments was therefore made in which the concentration of acid was varied from 1.0 N to about 4.1 N acid. The results are shown in Table V. The total acidity was determined approximately by titration. The values for the hydrogen ion concentration by the conductivity measurements were interpolated from Kohlrausch's tables. The hydrogen ion concentrations as determined by the E. M. F. method agree fairly well with those given by Noyes and MacInnes[9] except in the case of the strongest HCl concentration in which it is too low. It is remarkable that the rate of hydrolysis is in all cases nearly proportional to the hydrogen ion concentration as determined from the E. M. F. measurements. It is clear therefore that it cannot be the concentration of hydrogen ions as expressed in grams per liter which determines the rate of the reaction, since in the case of 3.7 N HCl it would be necessary to assume nearly 120 per cent dissociation. On the other hand, the result cannot be ascribed to the undissociated acid since it is hardly conceivable that the undissociated acid could effect the hydrogen electrode. It seems necessary to conclude that it is the "activity" of the hydrogen ion which determines the rate of hydrolysis of gelatin as well as the potential of the hydrogen electrode.

[17] Lewis, G. N., *Proc. Am. Acad. Arts and Sc.*, 1907, xliii, 259.

In other words, the "concentration" as determined by the hydrogen electrode is the effective concentration for the present reaction.

In a very recent paper Schreiner[18] has suggested the use of a special "catalysis-coefficient" to express the relation of the apparent hydrogen ion concentration, as determined by catalysis experiments, to the total acid concentration. Schreiner states that this coefficient is equal to the reciprocal of the conductivity ratio (corrected for

TABLE V.

Hydrolysis of 2.5 Per Cent Gelatin in Strong Acid at 40°C.

Acid.	Approximate concentration.	C_H E.M.F.	C_H Conductivity.	Velocity.	$K_H = \dfrac{\text{Velocity}}{C_H \text{ (E.M.F.)}}$	$\dfrac{\text{Velocity}}{C_H \text{ (conductivity)}}$
	N					
HCl.............	4.1	[5.5]	1.97	800	[130]	400
HCl.............	3.7	5.4	1.88	790	146	400
H₂SO₄..........	3.7	2.0	1.57	300	151	200
HCl.............	3.1	3.6	1.75	600	167	340
H₂SO₄..........	3.0	1.6	1.40	180	[125]	130
HCl.............	2.2	2.7	1.48	380	140	250
HCl.............	2.1	2.10	1.40	[400]	[190]	[280]
H₂SO₄..........	2.0	0.90	1.00	130	146	130
HCl.............	1.5	1.50	1.06	220	140	210
HCl.............	1.0	0.84	0.80	142	170	178
HCl.............	1.0	0.85	0.80	130	160	162
HCl.............	1.0	0.89	0.80	[160]	[180]	[200]
H₂SO₄..........	1.0	0.40	0.50	60	150	120
HCl.............	0.7	0.72	0.60	110	155	185
K_H = average............................					152	

viscosity), and he is able by its use to calculate quite closely the velocity of hydrolysis of methyl and ethyl acetate in concentrated acid or acid-salt mixtures. It seems unnecessary to introduce such a coefficient in order to account for the results of the experiments reported here, since it is possible to calculate the velocity, within the rather large experimental error, by means of the activity coefficient alone.

[18] Schreiner, E., *Z. anorg. u. allg. Chem.*, 1921, cxvi, 102.

III. Effect of Neutral Salts.

It was noted by Arrhenius[19] in 1899 that the addition of neutral salts to a weak acid increased the rate of hydrolysis of methyl acetate by the acid, which is just the opposite of the effect expected from the law of mass action. Arrhenius originally assumed that the salt actually increased the concentration of hydrogen ions. This view was criticised, however, and the hypothesis was put forward independently[20] by Dawson, and by Senter and Acree that the observed effect was due to the undissociated acid and a large number of experiments were performed which strengthened this view. It was found by Nelson and Fales,[21] however, that under certain conditions the effect of neutral salts on the activity of invertase could be ascribed entirely to the effect of the salt on the hydrogen ion concentration as measured by the hydrogen electrode. Since that time evidence has accumulated to show that the addition of neutral salts to a strong acid causes an increase in the hydrogen ion concentration as measured by the hydrogen electrode. A series of experiments was therefore performed to determine whether the addition of salts would influence the hydrolysis of gelatin. The results of this series are given in Table VI. It will be seen that the addition of 1.5 N NaCl to 1.0 N HCl solution increases the hydrogen ion concentration by nearly 50 per cent and that the rate of hydrolysis is increased practically the same amount. That is, if the effect of the neutral salt on the hydrogen ion concentration is taken into account, there is no effect on the rate of hydrolysis. In the case of NaCl and CaCl₂ the result is the same up to 1.0 N but above that the hydrogen ion concentration is increased more than the rate of hydrolysis. It was thought at first that this might be due to diffusion potentials caused by the NaCl or CaCl₂, since it will be noted that NaCl and CaCl₂ increase the hydrogen ion concentration more than the same concentration of KCl, whereas the effect on the rate of hydrolysis is the same for all the salts. (This is the result obtained

[19] Arrhenius, S., *Z. physik. Chem.*, 1899, xxxi, 197.

[20] For a review of this question see Lewis, W. C. McC., A system of physical chemistry, London, 1918–19, i.

[21] Fales, H. A., and Nelson, J. M., *J. Am. Chem. Soc.*, 1915, xxxvii, 2769.

TABLE VI.

Effect of Addition of Salts on Hydrolysis of 2.5 Per Cent Gelatin at 40°C.
HCl Concentration 1.0 N.

Salt.	Concentration.	pH	C_H E.M.F.	Velocity.	$K_H = \dfrac{\text{Velocity.}}{C_H}$
	N				
0...............	0	0.07	0.85	142	168
0...............	0	0.08	0.83	140	169
KCl............	1.0	+0.06	1.15	176	153
KCl............	1.0	+0.05	1.12	177	158
KCl............	1.5	+0.08	1.20	182	151
KCl............	1.5	+0.08	1.20	185	154
0...............	0	0.07	0.84	145	172
NaCl...........	0.5	+0.17	1.04	170	163
NaCl...........	1.0	+0.09	1.23	180⎱ 190⎰	⎧146 ⎨155
NaCl...........	1.5	+0.17	1.48	180	120
NaCl...........	1.65	+0.20	1.58	187	118
NaCl...........	1.65	+0.20	1.58	172	109
NaCl...........	1.65	+0.24	1.74	174	100
CaCl₂..........	0.5	+0.034	1.08	162	150
CaCl₂..........	1.0	+0.08	1.20	178	148
CaCl₂..........	1.5	+0.23	1.70	185	110

65°C. HCl Concentration 0.02 N.

Salt.	Concentration.	pH	C_H E.M.F.	Velocity.	$K_H = \dfrac{\text{Velocity.}}{C_H}$
0...............	0		0.0049	10	2.0×10^3
0...............	0		0.0045	11	2.4×10^3
KCl............	0.5		0.0043	10	2.3×10^3
KCl............	0.5		0.0043	13	3.0×10^3
KCl............	1.0		0.0043	12	2.8×10^3
KCl............	1.0		0.0045	10	2.2×10^3
KCl............	1.5		0.0041	12	2.9×10^3
KCl............	1.5		0.0041	12	2.9×10^3

by Arrhenius also.)   Measurement showed, however, that there was no diffusion potential in the case of NaCl at least. The diffusion potential was measured by determining the E. M. F. of the hydrogen electrode immersed in the NaCl-HCl solution, first against the sat-

urated KCl calomel electrode (in which case the hydrogen electrode in the NaCl-HCl is negative), and second, against another hydrogen electrode immersed in 1.0 N acid; a saturated KCl bridge was used in both cases. In this case the electrode in the NaCl solution is positive. Any diffusion potential therefore would tend to increase the measured potential in one case and decrease it in the other, so that the potential between the two hydrogen electrodes when measured directly should differ from the difference in potential of the two measured against the same calomel electrode by twice the value of the diffusion potential. The experiment showed, however, that an identical value was obtained by either method.

The experiments summarized in Table VI show that the effect of neutral salts can be accounted for by the increase of the hydrogen ion concentration. Whatever discrepancy is found is due to the fact that the hydrogen ion concentration apparently increased more than the rate of hydrolysis. It is evidently unnecessary to assume any activity of the unionized acid in this case at least.

Experiments in Low Concentration of Acid.—The above experiments were repeated with 0.02 N HCl (at 65°C.). In this case the addition of KCl has a very slight depressing effect on the hydrogen ion concentration and little if any influence on the rate of hydrolysis. This experiment also shows that the unionized gelatin chloride must hydrolyze at approximately the same rate as the ionized. It was shown by conductivity measurements[1] that gelatin chloride is practically completely ionized at pH 2.4, and that the addition of 1.0 N Cl ion reduces the ionization to a very small amount. It also decreases the rate of hydrolysis by pepsin very markedly and in direct proportion to the decrease in the conductivity of the gelatin chloride. The hydrolysis of gelatin by acid and by pepsin differs markedly in this respect. In pepsin hydrolysis the rate of digestion is decreased by increasing the amount of acid or salt beyond 0.01 N whereas in the acid hydrolysis the rate is unaffected by the addition of salt and increases in direct proportion to the hydrogen ion concentration with the addition of more acid. These facts agree with the hypothesis that the rate of hydrolysis of the ionized and non-ionized gelatin salt by hydrogen ions is the same, but that the ionized gelatin salt hydrolyzes much more rapidly than the non-ionized in the presence of pepsin.

IV. Influence of the Hydroxyl Ion Concentration.

It has been shown above that the velocity of hydrolysis of gelatin is directly proportional to the hydrogen ion concentration as measured

TABLE VII.

Hydrolysis of 2.5 Per Cent Gelatin in Strong Alkali at 40°C.

Alkali.	Concentration.	$C_{OH} = \dfrac{10^{-13.5}}{C_H}$.	Velocity.	$K_{OH} = \dfrac{\text{Velocity}}{C_{OH} \text{ (E.M.F.) } 25°C}$.
	N			
Ba(OH)₂.........	0.16	0.05	250	5,000
KOH.............	0.140	0.047	225	4,800
NaOH...........	0.12	0.045	230	5,100
KOH.............	0.69	0.30	1,000	3,300
NaOH...........	0.78	0.30	1,300	4,330
NaOH...........	1.0	0.45	2,250	5,000
KOH.............	1.27	0.60	2,300	3,900
NaOH...........	1.36	0.63	3,300	5,300
KOH.............	1.91	1.25	5,000	4,000
NaOH...........	2.0	0.90	6,000	6,600
NaOH...........	2.04	0.91	$\begin{bmatrix}8,000\\9,000\end{bmatrix}$	$\begin{bmatrix}9,000\\10,000\end{bmatrix}$
NaOH...........	2.72	1.20	10,000	$\begin{bmatrix}8,400\\7,100\end{bmatrix}$
NaOH...........	3.0	1.26	9,000	
KOH.............	2.55	2.0	8,500	4,250
KOH.............	3.82	3.2	17,000	5,300
NaOH...........	4.08	1.78	20,000	$\begin{bmatrix}11,000\\7,500\end{bmatrix}$
NaOH...........	4.1	1.86	14,000	
NaOH...........	4.9	2.70	21,000	7,800
Average...				4,700

by the hydrogen electrode in acid concentrations of from 0.10 to 4.1 N. In Table VII the results of a series of determinations in strong alkali are given. The concentration of hydroxyl ions was calculated

from the pH, taking the value for the dissociation constant of water at 40°C. as 2.8×10^{-14}. The rate is directly proportional in this range to the hydroxyl ion concentration as determined by the hydrogen electrode. It is not possible to compare the hydroxyl ion concentration as determined by the conductivity since the alkali solutions contained some carbonate. The constant is not so good here as in the acid solution since the reaction in strong alkali is extremely rapid. It will be noticed that the proportionality factor for the hydroxyl ion is 4700 whereas that for the hydrogen ion was 150; *i.e.*, the hydroxyl ions hydrolyze about 30 times more rapidly than hydrogen ions at the same concentration. It is not possible to say whether this difference is due to the hydrogen and hydroxyl ions or to the difference in the ease of hydrolysis of the gelatin-acid salt and the alkali gelatin.

V. *Influence of Hydrogen and Hydroxyl Ions in Low Concentration.*

It has been shown above that the velocity of hydrolysis in the acid range may be calculated from the equation

$$\text{Velocity}_H = 150 \, C_H$$

and in strongly alkaline solutions from the equation

$$\text{Velocity}_{OH} = 4700 \, C_{OH}$$

Combining these two equations we would calculate that (in round numbers) the velocity at any $C_H = 150 \, (C_H + 30 \, C_{OH})$. This is the same type of equation found by Wijs[22] to represent the influence of the concentration of hydrogen and hydroxyl ions on the rate of hydrolysis of methyl acetate. The formula predicts that the rate of hydrolysis is a minimum when the $C_H = 30 \, C_{OH}$ or at 40°C. at about pH 6.0. (The mathematical proof of this statement is that the first derivative of the above expression is zero at the point where $C_H = 30 \, C_{OH}$, and would be equal to approximately 3×10^{-4}; *i,e*, it would take nearly 30 years to cause an increase of 3 per cent in the formol titration of a gelatin solution at 40°C.) It was found, however, that the hydrolysis actually occurred at pH 6.0, about

[22] Wijs, J. J. A., *Z. physik. Chem.*, 1893, xii, 514.

300 times more rapidly than that predicted by the formula. The results of a series of experiments covering the range of from pH 1.0 to pH 12 are given in Table VIII and Fig. 5. In this figure the black circles are the experimental points; the line, the graph of the expression, Velocity = 150 (C_H + 30 C_{OH}); and the circles, the rate calculated from a formula considered below. It is necessary in this range to use some precaution to prevent the growth of microorganisms. Several different substances were used as indicated in the table. Control experiments were made at 65°C. on the influence of these
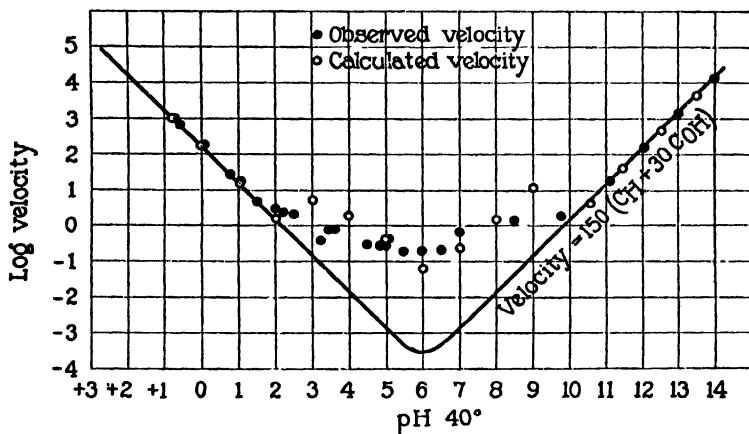
FIG. 5. Influence of the hydrogen ion concentration on the velocity of hydrolysis at 40°C.

on the rate of reaction—no significant effect was noted. In the case of $HgCl_2$ the pH was determined electrometrically before adding the $HgCl_2$ and then colorimetrically. It is a curious fact that nearly all disinfectants poison the hydrogen electrode. The experiment shows that the velocity of hydrolysis is directly proportional to the hydrogen ion concentration from pH 0.65 to pH 2.0. It then decreases much more slowly than the hydrogen ion concentration, passes through a very flat minimum at about pH 6.0 and then increases to become directly proportional to the hydroxyl ion concentration at about pII 10.0. The position of the minimum cannot be told with certainty from the figures given, since the difference

TABLE VIII.

Buffer.	Preservative.	40°C.	40°C.	Velocity.	$K_1 = \dfrac{\text{Velocity}}{C_H + 30\,C_{OH}}$	$K_2 = \dfrac{\text{Velocity}}{(C_H + 30\,C_{OH})(C_{uncombined} + 200\,C_{combined})}$
		pH	C_H			
See Table V......		+0.74	5.5 to 1.0	800–100	130–150	130–150
HCl.............		0	1	165	165	165
HCl.............		0.76	0.17	28	165	165
HCl.............		1.0	0.10	15	150	150
HCl.............		1.57	0.03	5	166	160
HCl.............	Toluene	1.80–1.9	$\left.\begin{array}{c}1.2\\1.6\end{array}\right\} \times 10^{-2}$	3.0	$\left.\begin{array}{c}180\\250\end{array}\right\}$	180
Citric..........	Toluene	2.20	6.3×10^{-3}	2.8	440	350
HCl citrate......	Benzoate	2.54	2.9×10^{-3}	2.4	830	30
Citrate-HCl......	HgCl₂	3.17	6.8×10^{-4}	0.4	600	150
Citrate-HCl......	Benzoate	3.60	2.5×10^{-4}	0.8	3,200	15
HCl citrate......	HgCl₂	4.50	3.1×10^{-5}	0.3	10,000	60
	HgCl₂	4.90	1.2×10^{-5}	0.3	21,000	120
Citrate HCl......	HgCl₂	4.90	1.2×10^{-5}	0.3	26,000	120
HCl.............	Toluene	4.9	1.2×10^{-5}	0.3	25,000	
	Toluene	5.01	1×10^{-5}	0.5	50,000	225
Citrate..........	HgCl₂	5.4	4×10^{-6}	$\left.\begin{array}{c}0.1\\0.2\end{array}\right\}$	31,200	150
Citrate..........	HgCl₂	6.0	1×10^{-6}		100,000	700
Citrate..........	HgCl₂	6.5	3.1×10^{-7}	0.2	64,000	450
			C_{OH} 40°C.			
Citrate-Na₂CO₃....	Benzoate	7.2	5×10^{-7}	0.2	12,000	90
	Benzoate	8.7–8.4	1.6–0.8×10^{-6}	1.8	$\left.\begin{array}{c}3,700\\1,850\end{array}\right\}$	150 75
Na₂CO₃..........	Thymol Toluene	9.75	1.8×10^{-4}	2.0	370	
NaOH, Na₂CO₃...		11.1	4×10^{-3}	18	150	150
NaOH, Na₂CO₃....	Benzoate	11.1	4×10^{-3}	20	165	165
NaOH..........		12.20	5×10^{-2}	250	165	165
NaOH..........		12.50	0.10	330	110	110
See Table VII....			0.05–3.2	250–1,700	160 ± 10	160

in the rates is not much greater than the experimental error. The determination of the minimum was checked, however, by noting the degree of liquefaction of the solutions after 90 days at 40°C. 10 cc. of the various solutions were brought to pH 5.0, the salt concentration and total volume made the same in all the tubes, and the tubes then immersed in a water bath at 5° for 2 hours. The degree of liquefaction was then noted. The result is given in Table IX. According to this determination the minimum point is about pH 6.5.

Two hypotheses may be suggested to explain this anomalous influence of low concentrations of hydrogen and hydroxyl ions. First, the velocity of hydrolysis is independent of the hydrogen ion concentration between pH 2.0 and 11.0 (or is proportional to the product of $C_H \times C_{OH}$). Second, some change takes place in the gelatin solution

TABLE IX.

pH	7.2	6.5	6.0	5.3	4.8
Liquefaction after 2 hours, 5°C	+	±	+	+++	++++

in this range which causes the gelatin to hydrolyze very much more rapidly and so compensate for the decrease in the C_H and C_{HO}.

It seems very unlikely from our knowledge of reactions in general that the rate of a reaction should be quantitatively proportional to the hydrogen ion concentration over a wide range and then suddenly become independent of it, to become later directly proportional to the hydroxyl ion concentration.

If we assume temporarily that the rate really is proportional to the hydrogen and hydroxyl ion concentration and that the discrepancy between the observed rate and the rate calculated on this basis is due to a change in the gelatin, we must assume that the gelatin is changed to a form which is very much more rapidly hydrolyzed. The value of the expression $\dfrac{observed\ velocity}{C_H + 30\ C_{OH}}$ will evidently be a measure of this change. In Fig. 6 the logarithms of the values of this expression are plotted over the pH together with the titration

curve of gelatin (taken from the titration curve for gelatin determined by Loeb), expressed as the per cent of uncombined gelatin present. It is apparent that the two curves are strikingly similar. Both are parallel to the X axis below pH 2 and then rise to a maximum, one at pH 5.0 and one at pH 6.0. Both then drop rapidly and again become parallel to the X axis beyond pH 11.0. This renders it probable that the rate of hydrolysis is some function of the amount of uncombined gelatin present. It will be noted from the table that the value of the "constant" at pH 5.0 (where the gelatin is completely uncombined) is about 200 times that of its value in the range where the gelatin is all present in the form of salt. If we assume then as a first approximation that the uncombined gelatin
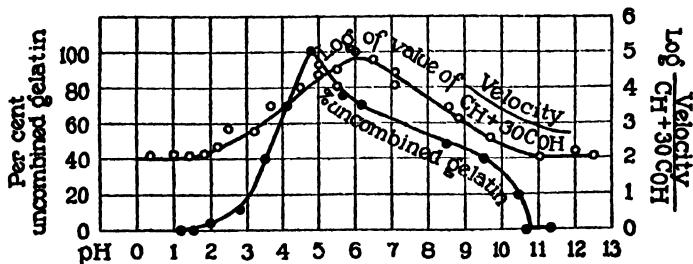
FIG. 6. Influence of the hydrogen ion concentration on percentage of uncombined gelatin and on the value of $K_1 = \dfrac{\text{Velocity}}{C_H + 30 C_{OH}}$.

hydrolyzes 200 times as rapidly as the combined and that the relative efficiency of the H and OH ions remains the same we get for the velocity of the reaction

$$\text{Velocity} = K_2 (C_H + 30\ C_{OH}) (C_{combined} + 200\ C_{free})\qquad(2)$$

where $C_{combined}$ and C_{free} refer to the concentration of free and combined gelatin as determined from the titration curve. The sum of these two quantities is taken as 1. The values for K_2 are given in the last column (Table VIII). It is evident that although they can hardly be considered as constant they are very much more so than the values for K_1. The rates calculated from (2) are plotted in Fig. 5. They may be considered satisfactory as a rough approximation.

It must be remembered that a slight error in the pH determinations causes a very large variation in the constant and that a shift of the steeper portions of the titration curves of less than 0.2 pH will make a difference in the value of K_2 of several hundred per cent. It must also be noted that the values on the figure are plotted as the logarithms so that the discrepancies are larger than they appear.

It may be pointed out that a reaction very similar to the above was studied by Clibbins and Francis[23] in connection with the hydrolysis of nitrosotriacetoneamine. In this case also the reaction is directly proportional to the C_{OH} over a wide range and then becomes nearly independent of it. In still more alkaline solution the velocity becomes inversely proportional to the C_{OH}. Clibbens and Francis consider also that this is due to some change in the condition of the substance undergoing the reaction but were unable to account for it quantitatively. Many reactions have been studied in which the velocity of reaction of the ions was very different from that of the free substance. Richards and Stull[14] found that, in the reaction between oxalic acid and bromine, the divalent oxalate ion reacts very much more rapidly than any of the other forms present. Stieglitz[24] accounted for the mechanism of the hydrolysis of the imido-esters by the hypothesis that the ester-salt hydrolyzed either more or less rapidly than the free ester. Similar explanations have been proposed by Acree, Goldschmidt, and others.

It will be noted that the peculiar results obtained in the present work are all in the range of acidity where the enzymes are active and where the growth of microorganisms is possible. It seemed possible, therefore, that the increased rate in this range might be due to traces of enzymes or to the presence of bacteria or the action of the various preservatives. The experiments were therefore repeated at a temperature of 65°, without the addition of preservatives. Any traces of enzymes present would be inactivated very rapidly at this temperature and their effect would be noticed only for the first few hours at most. No such effect was noted. The

[23] Clibbens, D. A., and Francis, F., *J. Chem. Soc.*, Tr. 1912, ci, 2358. Francis, F., and Geake, F. H., *Soc., J. Chem.* Tr. 1913, ciii, 1722.

[24] Stieglitz, J., *Am. Chem. J.*, 1908, xxxix, 29.

results of this series are given in Fig. 7. The pH measurements were made at 25° and calculated to 65° assuming the constant for water as 10^{-13} at this temperature and that the hydrogen ion concentration remains constant up to pH 8.0, and the hydroxyl ion concentration constant above pH 11.0. The curve is similar in every respect to that for 40°. There seems no possibility, therefore, that the anomalous rate of hydrolysis between pH 2.0 and 10.0 can be due to enzymes or microorganisms.

It will be noted that the rate of hydrolysis is 10 times more rapid at 65° than at 40° if solutions having the same hydrogen ion con-
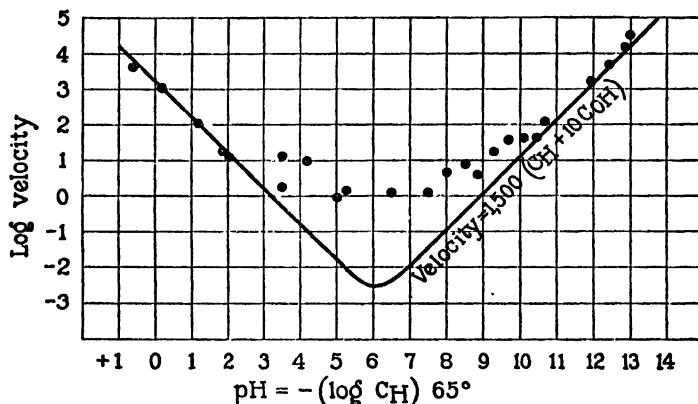
FIG. 7. Influence of the hydrogen ion concentration on the velocity of hydrolysis at 65°C.

centration at both temperatures are compared, but only 3 times more rapid if solutions having the same hydroxyl ion concentration are compared. This latter value seems remarkably low. A similar peculiarity was noticed by Michaelis and Rothstein[25] in studying the influence of the C_{OH} on the destruction of pepsin. These authors found that the destruction of pepsin was proportional to the third power of the C_{OH}, and that, if this was taken into account, the temperature had no effect on the velocity of the reaction.

Mechanism of the Reaction.—It was mentioned in the beginning of the paper that the hydrogen ion concentration decreases rapidly

[25] Michaelis, L., and Rothstein, W., *Biochem. Z.*,1920,cv. 60.

unless some means are provided such as buffer solutions for keeping it constant. The same is true for the hydroxyl ion concentration. According to the classical definition, therefore, the reaction is not catalytic since some of the catalyst combines with the products of the reaction and so would effect the final equilibrium. It seems probable that this is always true to some extent and that as was emphasized by Stieglitz, a "catalytic" reaction is merely the limiting case of an ordinary reaction in which the products of the reaction dissociate more or less completely liberating more or less of one of the original substances (cf. also Lewis[26] and Falk[27]). If the dissociation is complete no change could be detected in the concentration of one of the reacting substances and the reaction would be monomolecular in regard to the other. If there were no dissociation the reaction would, of course, be bimolecular. The present reaction is intermediate between the two. It is exactly analogous in this respect to the hydrolysis of gelatin by pepsin.

SUMMARY.

1. The hydrolysis of gelatin at a constant hydrogen ion concentration follows the course of a monomolecular reaction for about one-third of the reaction.

2. If the hydrogen ion concentration is not kept constant the amount of hydrolysis in certain ranges of acidity is proportional to the square root of the time (Schütz's rule).

3. The velocity of hydrolysis in strongly acid solution (pH less than 2.0) is directly proportional to the hydrogen ion concentration as determined by the hydrogen electrode i.e., the "activity;" it is not proportional to the hydrogen ion concentration as determined by the conductivity ratio.

4. The addition of neutral salts increases the velocity of hydrolysis and the hydrogen ion concentration (as determined by the hydrogen electrode) to approximately the same extent.

[26] Lewis, W. C., McC., A system of physical chemistry, 2nd ed., London. New York, Bombay, Calcutta, Madras, 1918–19, i, 416.

[27] Falk, K. G., The chemistry of enzyme actions, American Chemical Society Monograph Series, New York, 1921, 33.

5. The velocity in strongly alkaline solutions (pH greater than 10) is directly proportional to the hydroxyl ion concentration.

6. Between pH 2.0 and pH 10.0 the rate of hydrolysis is approximately constant and very much greater than would be calculated from the hydrogen and hydroxyl ion concentration. This may be roughly accounted for by the assumption that the uncombined gelatin hydrolyzes much more rapidly than the gelatin salt.

STUDIES ON MEASLES.

III. ACQUIRED IMMUNITY FOLLOWING EXPERIMENTAL MEASLES.

BY FRANCIS G. BLAKE, M.D., AND JAMES D. TRASK, JR., M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

It has been shown in a preceding paper[1] that monkeys (*Macacus rhesus*) inoculated intratracheally with unfiltered or filtered naso-pharyngeal washings from patients in the prodromal or early eruptive stage of measles exhibit an illness which closely resembles measles in man in its course and symptomatology. It has furthermore been shown[2] that the lesions which develop in the skin and buccal mucous membrane during the course of the infection in monkeys present essentially the same histologic picture that is found in the corresponding lesions of human measles. The experimental infection has been successfully transmitted[1] from monkey to monkey with the development of the same group of symptoms and pathologic lesions in the passage animals. These fundamental points of similarity between measles in man and the experimental disease in monkeys would appear sufficient to warrant the application of the term "experimental measles" to the latter condition. It has, nevertheless, seemed desirable to determine whether further points of resemblance between the two might not be shown.

Since an apparently permanent immunity against reinfection characteristically follows one attack of measles, the same phenomenon should hold true with respect to the experimental disease if the two conditions are to be regarded as similar. Furthermore, an acquired immunity, if present, should theoretically be efficient against a virus of heterologous source as well as against that of homologous origin, since there is little clinical evidence to show that one attack of measles fails to confer an immunity that is effective against all subsequent

---

[1] Blake, F. G., and Trask, J. D., Jr., *J. Exp. Med.*, 1921, xxxiii, 385.

[2] Blake, F. G., and Trask, J. D., Jr., *J. Exp. Med.*, 1921, xxxiii, 413.

exposures. Authentic reports of repeated attacks of measles in the same individual are so few as to be negligible in this connection. In order to test the validity of the foregoing assumptions a series of reinoculation experiments in monkeys which had recovered from a previous attack of experimental measles has been carried out as described below.

EXPERIMENTAL.

Six monkeys which had previously been inoculated with nasopharyngeal washings from cases of measles and had recovered from the ensuing attack of the experimental disease[3] have been subjected to reinoculation with material containing the virus of measles (Table I). In five instances virus of heterologous source was used, in one the homologous virus. In two monkeys the material, consisting of the supernatant fluid from an 0.85 per cent salt solution emulsion of the skin and buccal mucosa of a monkey killed on the 4th day of experimental measles, was injected intratracheally. In four monkeys whole blood withdrawn from a monkey on the 3rd day of experimental measles was injected intravenously. The intervals elapsing between recovery from the preceding experimental measles and the time of reinoculation varied from 12 to 254 days. None of the six monkeys following reinoculation showed any evidence of infection with the virus of measles, while the control normal monkeys, inoculated at the same time with equivalent amounts of the same material, developed the characteristic symptoms and pathologic lesions of the experimental disease. The protocols follow.

Experiment 1.—June 8, 1920. Monkeys 6, 9, and 19 were injected intratracheally at 12.15 p.m., 12.30 p.m., and 12.45 p.m., respectively, each with 10 cc. of the unfiltered supernatant fluid of an 0.85 per cent salt solution tissue emulsion (Virus MC. 3). The emulsion had been prepared from the minced and ground skin and buccal mucosa of Monkey 16, which was killed on the 4th day of experimental measles, about 24 hours after the first appearance of the exanthem. Monkey 6 had been inoculated Apr. 9, 1920, with pooled, filtered nasopharyngeal washings (Virus AM) from two sisters with measles and had recovered from the ensuing experimental measles on Apr. 23, 46 days before reinoculation. Monkey 9 had been inoculated May 12, 1920, with filtered nasopharyngeal washings (Virus

---

[3] For a detailed description of the first attack of experimental measles in these animals see Blake and Trask.[1]

MC) from a patient with measles and had recovered from the ensuing attack of experimental measles on May 27, 1920, 12 days before reinoculation. Monkey 19 was a normal monkey and served as a control.

TABLE I.

Immunity Following Experimental Measles.

Monkey No.	First inoculation.			Second inoculation.			
	Date.	Virus.	Result.	Date.	Interval after recovery from first attack.	Virus.	Result.
	1920			*1920*	*days*		
6	Apr. 9	AM	Experimental measles Apr. 16–22.	June 8	46	MC.3*	Remained well.
9	May 12	MC	Experimental measles May 20–26.	" 8	12	" 3	" "
19 (control).			.	" 8		" 3	Experimental measles, June 14–17. Killed.
2	Mar. 24	RG	Experimental measles Mar. 29–Apr. 4.	Dec. 15	254	JJ. 5	Remained well.
3	" 24	RK	Experimental measles Apr. 3–10.	" 15	248	" 5	" "
5	Apr. 9	AM	Experimental measles Apr. 16–22.	" 15	236	" 5	" "
8	May 12	MC	Experimental measles May 19–27.	" 15	201	" 5	" "
46 (control).				" 15		" 5	Experimental measles Dec. 18–27.

* The figure indicates the number of monkeys through which the virus had been passed.

Monkeys 6 and 9 showed no evidence of measles during 21 days observation. There were no conjunctivitis, no enanthem, and no exanthem, the eyes, buccal mucosa, and skin remaining normal in appearance throughout this period. Monkey 19 after an incubation period of 6 days developed the characteristic symptoms

of the experimental disease. On the 7th day a few discrete hyperemic spots appeared on the labial mucous membranes. On the 8th day the animal was listless; the conjunctivæ were injected; fresh Koplik spots had appeared on the mucous membrane of the cheeks. On the 9th day there was a well developed, confluent enanthem on the mucous membrane of the lips, gums, and cheeks. On the 10th day a few discrete, red maculopapules appeared about the lips, on the chin, and behind the ears. The animal was killed and the infection was successfully transmitted to two other monkeys. Histologic sections of the labial mucosa and tongue show the typical lesions of measles. The endothelial cells of the capillary walls are greatly swollen. There are a marked accumulation of endothelial leucocytes and some serous exudate about the capillaries, especially in the papillæ. In the stratified epithelium of the labial mucosa are many small foci (Koplik spots) showing endothelial leucocytes, serous exudate, and beginning necrosis of the epithelial cells. In some of these the process is more advanced and there is maceration of the epithelium, with shallow ulceration and secondary invasion by polymorphonuclear leucocytes. A few similar foci are seen in the epithelium of the tongue. There is also a more diffuse infiltration of the epithelium by endothelial leucocytes.

Experiment 2.—Dec. 15, 1920. Monkeys 2, 3, 5, 8, and 46 were injected intravenously in turn, each with 5 cc. of citrated whole blood (Virus JJ. 5) withdrawn from Monkey 36 on the 3rd day of experimental measles about 6 hours after the first appearance of the exanthem.

Monkey 2 had been inoculated Mar. 24, 1920, with nasopharyngeal washings (Virus RG) from a patient with measles and had recovered from the ensuing attack of experimental measles on Apr. 5, 254 days before reinoculation.

Monkey 3 had been inoculated Mar. 24, 1920, with nasopharyngeal washings (Virus RK) from a patient with measles and had recovered from the ensuing attack of experimental measles on Apr. 11, 248 days before reinoculation.

Monkey 5 had been inoculated Apr. 9, 1920, with pooled nasopharyngeal washings (Virus AM) from two cases of measles and had recovered from the ensuing attack of experimental measles on Apr. 23, 236 days before reinoculation.

Monkey 8 had been inoculated May 12, 1920, with nasopharyngeal washings (Virus MC) from a case of measles and had recovered from the ensuing attack of experimental measles on May 28, 201 days before reinoculation.

Monkey 46, normal, served as a control.

Monkeys 2, 3, 5, and 8 showed no evidence of infection during 21 days observation. They were well and active throughout this period. There were no conjunctivitis, no enanthem, and no exanthem. Monkey 46, after an incubation period of 3 days, developed the characteristic symptoms of measles. On the 4th day three Koplik spots appeared on the mucous membrane of the upper lip. On the 5th day the conjunctivæ were injected. On the 6th day a cluster of fresh Koplik spots was present on the mucous membrane of the lower lip. The animal was drowsy and listless. On the 7th day there were confluent patches of hyperemic enanthem studded with minute white specks on the labial mucosa. A few

red maculopapules appeared on the lower abdomen and inner surfaces of the thighs. By the 10th day there was a thick, red, maculopapular exanthem on the face, neck, chest, abdomen, and legs. By the 12th day the enanthem had faded; the exanthem was fading and showed fine branny desquamation. By the 14th day the animal had recovered except for slight remaining desquamation. Blood cultures on the 5th, 6th, and 7th days showed no growth. The infection was successfully transmitted from this animal to another monkey by means of blood withdrawn on the 5th, 6th, and 7th days. A section of skin excised[4] from the thigh on the 10th day shows the characteristic histologic picture of measles. About the capillaries and small veins in the upper layers of the corium there is a marked accumulation of endothelial leucocytes. Occasionally one is seen in mitosis. A few polymorphonuclear leucocytes are also present. Focal accumulations of endothelial leucocytes with vacuolation and necrosis of epithelial cells are seen in the epithelium of many of the hair sheaths and sebaceous glands. The epidermis shows vacuolation and necrosis of the cells of the Malpighian layer in minute foci. These areas are invaded by endothelial leucocytes. In the cornified layer are occasional, small, deeply staining plaques with the remains of minute vesicles beneath them.

<p style="text-align:center">DISCUSSION.</p>

The result of the foregoing experiments shows that one attack of experimental measles confers an apparently complete immunity against reinfection with measles for at least a considerable period. In all probability this immunity is permanent. In Experiment 1 it should be noted that Monkeys 6 and 9 were originally inoculated with filtered (Berkfeld N) nasopharyngeal washings. Their subsequent immunity, therefore, not only provides additional evidence of the similarity between human measles and the experimental disease but also tends to confirm the filterable nature of the virus. The strain of virus used in the reinoculation of Monkey 6 was of different origin from the strain with which this animal was originally inoculated, while with Monkey 9 the same strain of virus was employed in both the first and second inoculations. Since there was no apparent difference in the immunity of the two animals it would seem probable that the immunity provided by one attack of experimental measles is as efficient against a heterologous virus as against the homologous one. This is further supported by the result of the second experiment, in which four monkeys originally inoculated with strains of virus from four different sources exhibited a complete immunity against reinfection with a virus obtained from a still different source.

[4] This was done under ether anesthesia.

This result, as has been pointed out, was to be expected. Furthermore, it would suggest the probability that all strains of measles virus are of homologous nature in as far as their property of stimulating immunity is concerned, a fact which, of course, might readily be predicated from clinical observation.

The results of the two experiments, although they do not provide an explanation of the mechanism of acquired immunity against measles, nevertheless suggest certain possibilities. The course of measles, itself, in conjunction with evidence already presented[1] concerning the infectivity of the blood in the experimental disease, leaves little reason to doubt that the virus gains access to the blood by way of the respiratory mucous membrane and is subsequently distributed by the blood stream to the skin and buccal mucosa where it sets up the characteristic lesions of the disease. It is conceivable that the process of immunity against reinfection might reside in the respiratory mucous membrane which, in the immune animal, would present a barrier to invasion of the body by measles virus. While this supposition might serve as a possible explanation of the immunity exhibited by Monkeys 6 and 9 which were reinoculated by the intratracheal route, it is obviously inadequate in the case of the second experiment in which all the monkeys were inoculated intravenously. Since these animals showed an apparently complete immunity, it is clear that the immunity is not solely, if at all, dependent upon a possible barrier offered by the respiratory mucous membrane of the immune animal. That it is a function of the body tissues or fluids would seem more probable. Whether the immunity is humoral or cellular or both, however, only further experiment can determine.

SUMMARY.

It is shown that monkeys which have recovered from experimental measles are immune to reinfection with the virus of measles irrespective of whether the virus is of homologous or heterologous origin. In this respect experimental measles in the monkey corresponds with measles as observed in human beings, and the result is the same whether the virus is inoculated on the respiratory mucous membrane or is injected intravenously.

CONCLUSION.

Experimental measles in the monkey, like measles in man, is followed by an acquired immunity against the disease.

STUDIES ON BLOOD CHANGES IN PNEUMOCOCCUS INFECTIONS.

AN EXPERIMENTAL STUDY OF THE FORMATION AND FATE OF METHE-MOGLOBIN IN THE BLOOD.

BY WILLIAM C. STADIE, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

(Received for publication, January 27, 1921.)

INTRODUCTION.

Methemoglobin Formation in Pneumonia.

The pneumococcus, both *in vitro* (Gilbert and Fournier, 1896; Grüter, 1909; and Peabody, 1913) and *in vivo* (Butterfield and Peabody, 1913), has been shown to transform hemoglobin into methemoglobin. In consequence it seemed that methemoglobin formation might be the chief cause of the cyanosis sometimes observed in pneumonia. A study of the oxygen content and capacity of venous and arterial blood, however (Stadie, 1919), showed that the great and constant abnormality accompanying cyanosis was an increased proportion of reduced hemoglobin to oxyhemoglobin in the arterial blood. Therefore the essential cause of the cyanosis of pneumonia is incomplete oxygenation of the arterial blood, rather than the presence of methemoglobin in the blood. In only one of the thirty-two cases studied by the author was the oxygen capacity significantly reduced, as it would be if any considerable portion of the hemoglobin were changed to methemoglobin. On the contrary, in many cases the oxygen capacity of the blood was above the normal average.

Although we had but one case in which the oxygen capacity fell significantly, Peabody (1913) and Harrop (1919) have observed several cases in which such a fall occurred. Of eleven cases Peabody observed a marked fall of oxygen capacity in three, and Harrop in nine cases found a decrease of one-half in the total oxygen capacity in two. All these cases had positive blood cultures. In two other cases of Harrop's with negative blood cultures there was a decrease in oxygen capacity, but of less degree and over a greater period of time. It is possible that in these cases a considerable proportion of the hemoglobin may be altered into methemoglobin, and even in patients that do not show definitely lowered oxygen capacity small amounts of methemoglobin may be formed, and either be eliminated from the blood, or remain in it. In order to obtain more complete

evidence on these points a quantitative method for determination of methemoglobin was devised (Stadie, 1920), and in the present study it has been utilized in experimental work aimed to obtain evidence on the points in question.

General Conditions for Methemoglobin Formation.

The agents which change hemoglobin to methemoglobin are of varied nature. They include the following groups of known chemical substances: oxidizing substances, ozone, iodine, chlorates, ferricyanides, nitrites, nitrates, and azo compounds; reducing substances, pyrogallol, hydroquinone, hydroxylamine, etc.; organic bases, aniline, phenacetin, acetanilide, and toluidine; salts, sodium chloride in concentration above 1.5 per cent and ammonium sulfate in saturated solution. They also include several races of bacteria: *Streptococcus viridans*, cholera, pneumococcus, Gaertner bacillus, and certain nitrosobacilli (Wallis, 1913–14).

In vitro all these agencies produce methemoglobin with greater or less facility. Potassium ferricyanide rapidly forms methemoglobin from oxyhemoglobin. An amount of oxygen is liberated which is equivalent to the dissociable oxygen originally combined as oxyhemoglobin. Sodium nitrite converts hemoglobin to methemoglobin *in vitro*, but more slowly than potassium ferricyanide. The nitrite liberates an amount of oxygen equivalent to the amount necessary to change the nitrite to nitrate (Barcroft and Müller, 1911–12.) In other words, two molecules of nitrite transform one molecule of hemoglobin, producing one molecule of oxygen and one molecule of methemoglobin. Hydroxylamine transforms hemoglobin quantitatively, molecule for molecule (Letsche, 1912).

Of the above agents, we have used in our experiments potassium ferricyanide and sodium nitrite.

Characteristics of Clinical and Experimental Methemoglobinemia.

Agents.—Human cases of methemoglobinemia are the result of poisoning with various agents, of which aniline is perhaps the most familiar. In laboratory animals methemoglobin is produced easily by nitrites, potassium ferricyanide, acetanilide, and the pneumococcus.

Symptoms.—In the overwhelming methemoglobinemia produced experimentally it is difficult to dissociate the symptoms produced by

the drug from those produced by the methemoglobin. Rapid breathing and air-hunger are constant symptoms in severe grades of methemoglobinemia, and when 70 to 80 per cent of the hemoglobin is changed the animal exhibits all the signs of acute suffocation and dies in a short time. In milder instances with a destruction of 25 to 50 per cent of the hemoglobin, usually there are no symptoms in rabbits. It is doubtful whether the mere presence of large amounts of methemoglobin in the body is harmful.

Cyanosis is a regular accompaniment of methemoglobinemia. This, of course, is due to the fact that the presence of methemoglobin in blood gives it a dark color closely resembling that of venous blood. Transformation of as little as 5 per cent of the hemoglobin to methemoglobin in blood gives it a dark color. The blood does not become bright red on exposure to the air and is easily distinguishable from normal oxygenated blood. The cyanosis itself is indistinguishable from the cyanosis due to oxygen unsaturation of the arterial blood. Two important differences, however, may be noted: (1) the cyanosis of oxygen unsaturation usually accompanies pulmonary or heart disease and varies in intensity with change of position, coughing, and exertion; (2) the administration of oxygen may diminish this cyanosis. The final differentiation is made by the spectroscope or by methemoglobin determination, for which a method has been recently published (Stadie, 1920).

Pathology.—No characteristic changes of the organs have been described in animals in which a severe methemoglobinemia has been induced. Certain hemorrhagic changes and areas of necrosis in the liver and spleen have been described, but these have been inconstant and have followed large doses of potassium chlorate, and consequently might easily have been due to this substance rather than to the methemoglobin.

The proportion of hemoglobin changed may be as great as 100 per cent, as after the intravenous injection of sodium nitrite in rabbits. With acetanilide or sodium nitrite in proper doses a reduction in the amount of hemoglobin of 60 to 70 per cent may be brought about easily. The animals often recover; the sudden flooding of the body by so large an amount of methemoglobin is without apparent permanent effect.

Methemoglobin may exist in the blood in two distinct conditions. First, it may be present in the plasma alone, a true methemoglobinemia. This is, however, rare, but is illustrated by Brandenburg's case of potassium chlorate poisoning which showed a rapid decrease of red blood cells from 4,300,000 to 1,600,000 in 6 days. The serum showed methemoglobin spectroscopically. Second, the methemoglobin is present within the red blood cells—a condition of methemoglobincythemia. This is the usual occurrence. Cases of nitrite, acetanilide, and nitrobenzene poisoning and bacterial infections with methemoglobin formers fall into this class.

EXPERIMENTAL.

Fate of Methemoglobin.—Methemoglobin solutions injected intravenously are rapidly eliminated from the blood. Table I shows

TABLE I.

Elimination of Methemoglobin Following Intravenous Injection in Rabbits.

Rabbit No.	Time.	Injection.	Hemo-globin per 100 cc. of blood.	Methemoglobin spectrum.		
				Serum.	Cells.	Urine.
	p.m.		*gm.*			
1	12.10		13.87			
	12.20	2 gm. of methemoglobin intravenously.				
	12.45		12.04	Negative.	Negative.	
	4.00	(Killed.)*	11.87	"	"	++++
	a.m.					
2	9.05		10.03			
	11.00	1.7 gm. of methemoglobin intravenously.				
	11.15		10.03	Negative.	Negative.	++++

*Extracts of lungs, liver, spleen, heart, feces, and intestines showed no methemoglobin spectroscopically.

the extreme rapidity with which methemoglobin dissolved in the plasma disappears. The hemoglobin was determined gasometrically by Van Slyke's method (1918), the methemoglobin by the author's method (1920).

Within 15 to 25 minutes an amount of methemoglobin equal to 20 to 22 per cent of the total hemoglobin was completely removed from

the blood so that none could be found in it spectroscopically. At least part of the methemoglobin was excreted in the urine.

Storage of Methemoglobin.—Extracts of the various organs made immediately post mortem revealed no evidence that methemoglobin is accumulated in any one place. When the methemoglobin is present in the red blood cells only, even when all the hemoglobin is changed to methemoglobin, with death by virtual suffocation due to lack of labile oxygen, the plasma is always free from methemoglobin bands.

The methemoglobin produced in the red blood cells is rapidly destroyed. In the milder cases of experimental methemoglobin-cythemia this destruction was found to be so rapid that repeated and careful examinations of blood cells, plasma, urine, and tissues failed to reveal its presence.

Table II gives the results from rabbits injected with solutions of potassium ferricyanide. The action of this substance *in vivo* is relatively slow, but by repeated injections over a period of several hours almost 50 per cent of the hemoglobin may be changed. In the three rabbits the potassium ferricyanide changed 43, 30, and 19 per cent respectively of the hemoglobin, doubtless into methemoglobin, and yet the latter was so rapidly destroyed that practically none of it could be found in the blood.

In another rabbit (Table III) within 5 days the hemoglobin diminished 39 per cent, yet no methemoglobin was found spectroscopically in either plasma or cells. Moreover, the urine and the aqueous extracts of liver, lungs, spleen, kidney, heart, skeletal muscle, intestines, and feces showed no methemoglobin by spectroscope.

The rapid production of methemoglobin, as by the injection of sodium nitrite, by which an easily controllable degree of methemoglobin formation may be brought about gives the same results.

In Rabbit 7 (Table IV) practically all the hemoglobin was changed to methemoglobin, the animal dying immediately of suffocation. The pigment is wholly in the cells. Rabbit 8 lived for 26 minutes with hemoglobin of only 2.9 gm. per 100 cc. of blood, or 23 per cent of the initial value. Yet within this short time the methemoglobin had decreased to 6.7 gm. per 100 cc. of blood. A rabbit of 2.85 kilos has 142 cc. of blood (5 per cent by weight (Van Slyke and Salvesen, 1919)), or in this case 18.1 gm. of hemoglobin, of which at death 4.1

<div align="center">

TABLE II.

Production of Methemoglobin in Rabbits by Potassium Ferricyanide.

</div>

Rabbit No.	Date.	Time.	Injection.	Hemoglobin per 100 cc. of blood.	Methemoglobin per 100 cc. of blood.
	1919	*a.m.*		*gm.*	*gm.*
3	Apr. 25	9.30		15.2	
		10.15	10 cc. of 0.02 M potassium ferricyanide intravenously.		
		10.30		13.2	
		11.50	10 cc. of 0.02 M potassium ferricyanide intravenously.		
		p.m.			
		12.15		11.0	
		2.45	5 cc. of 0.02 M potassium ferricyanide intravenously.		
		3.15		8.8	
	Apr. 29			8.9	·
	May 6			8.7	0.0
		a.m.			
4	May 7	11.35		11.1	
		11.45	5 cc. of 0.02 M potassium ferricyanide intravenously.		
		p.m.			
		12.45		10.5	0.0
		1.02	5 cc. of 0.02 M potassium ferricyanide intravenously.		
		2.15		10.0	0.3
		2.30	5 cc. of 0.02 M potassium ferricyanide intravenously.		
		2.31	(Animal died.)	7.8	0.2
		a.m.			
5	May 9	9.20		13.3	
		9.40	20 cc. of 0.02 M potassium ferricyanide intravenously.		
		9.45		14.1	0.0
		10.30		13.0	0.4
		11.45		12.5	0.0
		p.m.			
		2.07	10 cc. of 0.02 M potassium ferricyanide intravenously.		
		2.30		10.8	0.0

TABLE III.

Production of Methemoglobin in Rabbits by Potassium Ferricyanide.

Rabbit No.	Date.	Time.	Injection.	Hemoglobin per 100 cc. of blood.	Methemoglobin per 100 cc. of blood.	Methemoglobin spectrum. Plasma.	Cells.
				gm.	*gm.*		
6	*1919* June 5	*m.* 12.00	10 cc. of 0.02 M potassium ferricyanide intravenously.	11.6*			
		p.m. 2.27					
	June 6	*a.m.* 10.00	5 " " 0.02 M "				
		11.07	5 " " 0.02 M "				
		11.47	5 " " 0.02 M "				
		f.m. 12.21	5 " " 0.02 M "				
		1.45	5 " " 0.02 M "				
		2.47	5 " " 0.02 M "				
		4.00	5 " " 0.02 M "				
	June 7	*a.m.* 10.58	5 " " 0.02 M "				
		11.45	5 " " 0.02 M "				
	June 9	*p.m.* 1.15	5 " " 0.02 M "				
		3.40	10 " " 0.02 M "	8.2	0.2		
		4.32	10 " " 0.02 M "				
	June 11	4.30	(Killed.)†	7.0	0.5	Negative.	Negative.

* Colorimetric determination.

† Extract of liver, lungs, spleen, heart, skeletal muscle, intestines, and feces showed no methemoglobin spectrum. Urine negative also.

gm. were left. Of the 14 gm. of methemoglobin formed, only 9.5 gm. remained at death. In other words, 4.4 gm. of methemoglobin were destroyed in 26 minutes. In Rabbit 9, 1.8 gm. of methemoglobin were destroyed in 30 minutes. In all these rabbits no methemoglobin was found in the plasma, although the blood was dark chocolate in color; it was present in the cells only.

Therefore even in extreme cases of methemoglobin production in which death results quickly from an insufficient oxygen supply, there is a rapid destruction of methemoglobin. When the methemoglobin is produced more slowly this destruction is fast enough to prevent its accumulating in the blood in sufficient quantity to be detected by the spectroscope. Not only is this true when chemicals are the causative agent, but also when methemoglobin-producing bacteria act *in vivo*.

However, when the production of methemoglobin is very extensive, *e.g.* 30 to 50 per cent of total pigment, and sudden, as following intravenous injections of sodium nitrite, methemoglobin may be found in the blood, but always in the red blood cells. Even in these instances, if the animal survives for a comparatively brief time, the methemoglobin disappears from the blood.

The mechanism of this disappearance has not been determined. Pearce, Austin, and Eisenbrey (1912) studied the fate of hemoglobin injected intravenously and found that no hemoglobin was eliminated through the kidney unless the rate of injection was above a minimum which was high, but that nevertheless the injected hemoglobin rapidly disappeared from the blood stream. Furthermore, Whipple and Hooper (1913) showed that injected hemoglobin is changed rapidly to bile pigments in normal dogs and in dogs with Eck fistula or hepatic ligation. Since methemoglobin is closely related chemically to hemoglobin it is possible that it is similarly disposed of.

Action of Pneumococci on Hemoglobin in Vivo.—Grüter (1909) inoculated a cat intraperitoneally with a large amount of pneumococcus culture but was unable to demonstrate methemoglobin in the blood. Peabody (1913) developed the subject further by inoculating rabbits intravenously with the organisms from 300 to 600 cc. of 24 hour broth cultures of pneumococci. Death resulted within a few hours and the₊blood always showed on direct film enormous num-

TABLE IV.

Intravenous Injection of Sodium Nitrite in Rabbits. Rapid Formation and Disappearance of Methemoglobin.

Rabbit No.	Weight.	Time.	Injection.	Hemoglobin per 100 cc. of blood.	Methemoglobin per 100 cc. of blood.	Methemoglobin* destroyed.	Methemoglobin spectrum.		
							Plasma.	Cells.	Urine.
	kg.	*a.m.*		*gm.*	*gm.*	*gm.*			
7		9.15		7.8					
		9.30	0.95 gm. of sodium nitrite intravenously.				Negative.	++++	Negative.
		9.31	(Died.)	0.3					
		p.m.							
8	2.85	3.00		12.7	0.0				
		3.21	0.1 gm. of sodium nitrite per kilo intravenously.				Negative.	++++	Negative.
		3.47	(Died.)	2.9	6.7	4.4			
		a.m.							
9	1.7	9.00		10.0	0.0				
		9.30	0.12 gm. of sodium nitrite per kilo intravenously.				Negative.	++++	Negative.
		10.00	(Died.)	3.6	4.3	1.8			

*Estimated on the assumption that the blood volume is 5 cc. per 100 gm. of body weight (Van Slyke and Salvesen, 1919).

bers of pneumococci. With these overwhelming pneumococcemias Peabody showed that there was a rapid fall in total hemoglobin as measured by oxygen capacity, but he was rarely able to demonstrate the presence of methemoglobin in the blood. He nevertheless concluded that this fall in total oxygen capacity (hemoglobin) was due to a methemoglobin production. However, when these experiments are repeated with quantitative determination of methemoglobin, it is clear that bacterial methemoglobinemia is similar to that produced chemically in that the methemoglobin formed rapidly disappears.

Rabbits were inoculated by ear vein with the centrifuged pneumococci from 24 hour cultures resuspended in a small amount of saline solution. Death occurred in all cases and all showed by direct film of the blood enormous numbers of organisms. The results are shown in Table V.

In Rabbit 10, after 5 hours, despite a fall in hemoglobin of 3.94 gm. per 100 cc. of blood (30 per cent of the total), there was no methemoglobin in the blood. In No. 12, 3.82 gm. of hemoglobin per 100 cc. of blood (30 per cent of the total) were changed, but only 0.83 gm. of methemoglobin per 100 cc. was found. In Nos. 10 and 11 at death considerable amounts of methemoglobin were found in the blood, but amounts representing only 30 to 32 per cent of the total change. It is clear then that the mechanism here is the same that is described in the production of methemoglobin by chemicals. In the gradual change of hemoglobin to methemoglobin the latter pigment disappears as fast as it is formed, so that it is never present in the blood in sufficient concentration to be detected spectroscopically. Only near death does the rapid production of methemoglobin by the huge numbers of pneumococci lead to its accumulation in the blood in quantities greater than the now disordered metabolism can handle. In other words, it is rarely possible to find methemoglobin in the blood, unless just before death, even after considerable changes of hemoglobin to methemoglobin.

It is to be further noted that the methemoglobin present in these instances is entirely intracellular. Examination of the plasma spectroscopically gives negative results, but the centrifuged cells in the last samples of Nos. 11 and 12 contain abundant methemoglobin. The urine also is always negative. The condition is a methemoglobincythemia.

TABLE V.

Production of Methemoglobin by Pneumococci in Rabbits.

Rabbit No.	Date.	Time.	Conditions.	Hemoglobin per 100 cc. of blood.	Hemoglobin decrease per 100 cc. of blood.	Methemoglobin per 100 cc. of blood.	Remarks.
	1919	*a.m.*		*gm.*	*gm.*	*gm.*	
10	June 15	11.45		12.98	0.0	0.0	
		p.m.					
		1.00	Inoculated.				
		3.30		10.82	2.16	0.30	Blood bright red.
		5.00		9.04	3.94	0.00	" " "
		8.45	Very sick.	6.02	6.96	2.09	" chocolate-colored.
		9.00	Died.				
		a.m.					
11	June 25	9.00		12.78		0.0	
		10.25	Inoculated.				
	June 26	10.25		11.60	1.18	0.18	Blood bright red.
		p.m.					
		4.19	Died.	5.75	7.03	2.27	" chocolate-colored.
		a.m.					
12	June 30	9.00		12.87		0.0	
		10.50	Inoculated.				
		p.m.					
		3.30	Died.	9.05	3.82	0.83	Blood bright red.

Rabbit 10 received Pneumococci Type I from 250 cc. of broth cultures. Rabbit 11 received Pneumococci Type I from 100 cc. of broth cultures. Rabbit 12 received Pneumococci Type II from 150 cc. of broth cultures.. Postmortem films of blood from all the rabbits showed enormous numbers of pneumococci.

Non-Production of Methemoglobin by Pneumococcus Autolysates.

Grüter (1909) observed methemoglobin formation in the presence of dead cultures, filtrates, and centrifugates. Butterfield and Peabody (1913) also have produced methemoglobin by the autolysates of pneumococcus cultures. Rieke (1904), however, attributed methemoglobin formation to the living pneumococcus and streptococcus only. Cole (1914) likewise reached the same conclusion, since he was unable to demonstrate the production of methemoglobin by filtrates and extracts of pneumococcus cultures.

We have also been unable to show a transformation of hemoglobin to methemoglobin without the living organism. A broth culture of pneumococcus was used in which, after 5 days incubation, all organisms were autolyzed as shown by sterile subcultures. Prepared in this way the pneumococcus autolysate failed to produce methemoglobin when mixed with hemoglobin solutions, as shown in Table VI.

It is quite possible, however, that if, as has been suggested by Avery and Cullen,[1] the proper conditions are found for the reaction or if concentrated solutions of intracellular products obtained by the disintegration of the pneumococcus are used, methemoglobin formation without the living organism may be demonstrated.

TABLE VI.

Action of Autolysate of Pneumococci on Hemoglobin.

Tube No.	Conditions.	Broth.	Autoly-sate.	Hemo-globin solution.	Initial hemo-globin.	Hemo-globin after 20 hrs.	Methemo-globin after 20 hrs.
		cc.	*cc.*	*cc.*	*per cent*	*per cent*	*per cent*
1	Room temperature......	0	10	10	7.14	6.45	0.69
2	Ice box...............	0	10	10	7.14	7.14	0.00
3	Room temperature......	10		10	7.30	6.67	0.63

DISCUSSION.

Methemoglobin Formation in Pneumonia.—Peabody (1913) was the first to explain fall of blood oxygen capacity observed in some (usually fatal) pneumonia cases as due to a change of hemoglobin to methemoglobin. He suggested that this alteration could take place through the action of a soluble product of bacterial metabolism. If the production of methemoglobin by soluble bacterial products, as suggested by Peabody, is substantiated by further experiments, then it is possible that the decrease in hemoglobin may be brought about by the escape of these products into the blood stream from a focus of pneumococcic infection in the lungs. However, all the cases studied by Peabody (1913) and Harrop (1919) with a sudden and well defined decrease of hemoglobin had a pneumococcemia of profound degree (from 2,500 to 16,000 colonies per cc. of blood). It is, therefore,

[1] Avery, O. T., and Cullen, G. E., unpublished work.

probable that a great production of methemoglobin occurs only when the opportunity for the direct action of the pneumococci on the blood is greatest; *i.e.*, in cases with a bacteremia.

Methemoglobin and Cyanosis in Pneumonia.—Although there has been a prevalent belief that methemoglobinemia is a factor in the cyanosis of pneumonia, many observers (Abrahams, Hallows, and French, 1919; Synnott and Clark, 1918; and Peabody, 1913) have looked for it spectroscopically in cases of pneumonia deeply cyanotic, but have failed to find it.

The absence of methemoglobinemia in pneumonia, even when there is a marked fall in oxygen capacity, is, we believe, explainable by the results reported in this paper. Methemoglobin disappears from the circulation with great rapidity, whether it is introduced by injection of methemoglobin or formed within the circulation by the action of chemicals or of pneumococci. If we take into consideration this fact, the ability of the pneumococcus to form methemoglobin, and the consistent failure to find it in the blood in the cyanosis of pneumonia, it appears that the following conclusions represent the most probable explanation of what occurs.

CONCLUSIONS.

In the occasional cases of pneumonia which show a decrease in the oxygen capacity of the blood, the decrease is probably due to a formation of methemoglobin. The latter is removed from the circulation, however, as rapidly as it is formed, so that it can seldom be detected even qualitatively, and is probably never a cause of cyanosis.

BIBLIOGRAPHY.

Abrahams, A., Hallows, N., and French, H., *Lancet*, 1919, i, 1.

Barcroft, J., and Müller, F., *J. Physiol.*, 1911–12, xliii, p. xx.

Blake, F. G., *J. Exp. Med.*, 1916, xxiv, 315.

Boycott, A. E., *Brit. Med. J.*, 1911, ii, suppl., 409.

Brandenburg, cited by Oettinger, B., *J. Am. Med. Assn.*, 1905, xlv, 826.

Buckmaster, G. A., *J. Physiol.*, 1914, xlviii, p. xxv.

Butterfield, E. E., and Peabody, F. W., *J. Exp. Med.*, 1913, xvii, 587.

Cole, R., *J. Exp. Med.*, 1914, xx, 363.

Dennig, A., *Deutsch. Arch. klin. Med.*, 1899–1900, lxv, 524.

Dittrich, P., *Arch. exp. Path. u. Pharmakol.*, 1891–92, xxix, 247, 251.

Filehne, W., *Arch. exp. Path. u. Pharmakol.*, 1878, ix, 329.

Gibson, G. A., *Quart. J. Med.*, 1907–08, i, 29.

Gilbert, A., and Fournier, L., *Compt. rend. Soc. biol.*, 1896, xlviii, 2.

Grüter, W., *Centr. Bakt., 1te Abt., Orig.*, 1909, 1, 241.

Harrop, G. A., Jr., *Bull. Johns Hopkins Hosp.*, 1919, xxx, 10.

Hayam, G., *Compt. rend. Acad.*, 1886, cii, 698.

Heubner, W., *Arch. exp. Path. u. Pharmakol.*, 1913, lxxii, 239.

Hoppe-Seyler, F., *Centr. med. Wissensch.*, 1864, ii, 834.

Hoppe-Seyler, F., *Z. physiol. Chem.*, 1877–78, i, 396.

Kobert, R., *Arch. ges. Physiol.*, 1900, lxxxii, 615.

Letsche, E., *Z. physiol. Chem.*, 1912, lxxx, 412.

Lundsgaard, C., *J. Exp. Med.*, 1919, xxx, 147, 259, 271, 295.

Peabody, F. W., *J. Exp. Med.*, 1913, xviii, 1, 7.

Pearce, R. M., Austin, J. H., and Eisenbrey, A. B., *J. Exp. Med.*, 1912, xvi, 375.

von Rheinbold, B., *Z. physiol. Chem.*, 1913, lxxxv, 250.

Rieke, H., *Centr. Bakt., 1te Abt., Orig.*, 1904, xxxvi, 321.

Stadie, W. C., *J. Exp. Med.*, 1919, xxx, 215.

Stadie, W. C., *J. Biol. Chem.*, 1920, xli, 237.

Synnott, M. J., and Clark, E., *J. Am. Med. Assn.*, 1918, lxxi, 1816.

Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxiii, 127.

Van Slyke, D. D., and Salvesen, H. A., *J. Biol. Chem.*, 1919, xl, 103.

Wallis, R. L. M., *Quart. J. Med.*, 1913–14, vii, 73.

Whipple, C. H., and Hooper, C. W., *J. Exp. Med.*, 1913, xvii, 612.

Ziemke, E., and Müller, F., *Arch. Physiol.*, 1901, suppl., 177.

STUDIES ON BACTERIAL NUTRITION.

I. GROWTH OF BACILLUS INFLUENZÆ IN HEMOGLOBIN-FREE MEDIA.

BY THEODOR THJÖTTA, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

(Received for publication, March 8, 1921.)

Pfeiffer in 1892 first obtained growth of *B. influenzæ* by the use of agar slants, the surface of which was smeared with a few drops of human blood. Pfeiffer showed that this bacillus grew only in media containing blood or hemoglobin. The influenza bacillus was thus brought into the group of the hemoglobinophilic bacilli and has since always been considered an obligate hemophilic organism despite the fact that the literature shows that several investigators have been able to cultivate it in media free of blood or hemoglobin.

The first to show this fact was Cantani (1), who in 1901 obtained growth of *B. influenzæ* on ascites agar in symbiosis with other bacteria, such as gonococcus, diphtheria bacillus, and several large cocci. *B. influenzæ* grew in giant colonies in the vicinity of the other colonies. Cantani also obtained growth on the surface of agar to which had been added emulsions of bacteria killed by heat at 60°C. for 3 hours. Cantani excludes symbiosis and supposes that some factor in the bacterial cell induces the growth of *B. influenzæ* and that this factor is more easily liberated from the dead than from the living cell.

Neisser (2), however, demonstrated the growth of influenza bacilli in symbiosis with xerosis bacilli on plate cultures made from the inflamed conjunctivæ of a child. Impure colonies were observed which could be transferred through many generations on plain agar without the disappearance of *B. influenzæ*. The latter, however, failed to grow on this medium if the xerosis bacillus was not present.

Neisser also tried to cultivate *B. influenzæ* on media prepared by adding killed emulsions of the xerosis bacillus or the diphtheria bacillus to plain agar. A slight growth occurred for three or four generations, but the continued cultivation of *B. influenzæ* on this medium was impossible. He therefore considered the growth of *B. influenzæ* in mixed cultures as a symbiotic phenomenon.

Grassberger (3) observed that when *B. influenzæ* was grown on blood or hemoglobin agar plates in association with other bacteria, especially staphylococci, the colonies of influenza bacilli adjacent to the colonies of cocci were of unusually large size. He assumed that the cocci exerted some effect upon the blood medium that was beneficial for the growth of *B. influenzæ*.

Luerssen (4) confirmed the observations of Cantani by growing *B. influenzæ* on agar containing emulsions of dead staphylococci, *B. coli,* or *B. prodigiosus.* Growth of *B. influenzæ* did not occur in ordinary mixed cultures with these other organisms. The bacterial emulsions were sterilized by heating at 60°C. for 3 hours; if they were boiled the growth which occurred was not so good. Luerssen presumes that the bacteria contain a factor that exerts a stimulating effect upon the growth of *B. influenzæ*, and that this is destroyed at high temperatures. He found that this growth factor is contained in the bacterial cell, since the carefully washed cells are still active. Growth of *B. influenzæ* did not occur if the enriching emulsion of dead organisms was smeared on the surface of the agar; it only occurred if the emulsion was incorporated in the medium. Luerssen also observed that *B. influenzæ* grew sparsely in sterile filtrates of broth cultures of staphylococci, *B. diphtheriæ*, and *B. violaceus.*

Ghon and von Preyss (5) attempted to grow *B. influenzæ* in media containing no hemoglobin, but the results were negative and they concluded that the reason other investigators had been successful was that in making the inoculations small amounts of blood had been carried over. They likewise held the opinion that when *B. influenzæ* grows on plain agar, as occasionally happens, this agar contains traces of hemoglobin.

Recently Putnam and Gay (6) tried to confirm the experiments of the earlier investigators. They were unable, however, to obtain any growth of *B. influenzæ* on plain agar to which either killed or living cultures of *B. xerosis, B. diphtheriæ, B. coli,* or staphylococci had been added.

In spite of the observations concerning the growth of *Bacillus influenzæ* in media free of blood or hemoglobin, the opinion that this organism is hemoglobinophilic has not been altered, and for its cultivation media containing blood or hemoglobin in some form is always employed.

EXPERIMENTAL.

In a study on the growth of mucoid bacilli and the transformation of non-mucoid bacilli into mucoid ones (7) the writer undertook the cultivation of different microbes, *Bacillus paratyphosus* B and pneumococci, in broth containing mucus produced by mucoid bacilli such as Friedländer's bacillus, the ozenabacillus, and other similar organisms. An attempt was also made to cultivate *Bacillus influenzæ* in this medium. The development of this work led to the present studies on bacterial nutrition.

Experiment 1.—A culture of a mucoid Gram-negative bacillus (Friedländer's) was grown on plain agar. After 24 hours in the incubator the growth from each plate was suspended in a few drops of plain broth. 0.5 cc. of this emulsion was added to 5 cc. of plain broth, then heated for 1 hour at 60°C., tested for sterility, and stored in the ice box. The reaction of the broth, pH 7.8, was not altered by the addition of the bacterial emulsion. With the medium prepared in this manner, the following tests were made.

A tube of this medium was inoculated with influenza bacilli from a blood broth culture, the blood cells of which had completely settled to the bottom of the

TABLE I.

Growth of B. influenzæ in Plain Broth Added to Emulsions of Friedländer's Pneumonia Bacillus.

No. of transfers from original culture in emulsion broth.	Age of culture when transferred.	Growth of culture on blood agar after standing in incubator. No. of days.									
		1	2	3	4	5	6	7	8	9	10
Emulsion broth 1	*hrs.* 48	++	++				0				0
" " 2	*days* 4				+						+
" " 3	*hrs.* 24	++							+		
" " 4	48	++			++			++			
" " 5	48	++			++						0
" " 6	*days* 5	++	++								0
" " 7	*hrs.* 24	++			++						
" " 8	48	++			++						
" " 9	Not transferred.	++									

tube. The following day 0.2 cc. of this subculture was transferred to another tube of emulsion broth. Simultaneously a loopful of the first culture was streaked on the surface of blood agar to demonstrate the growth of *B. influenzæ*. The last procedure was necessary since the emulsion broth in itself was too cloudy to show growth. From the first culture there were made eight successive transfers to emulsion broth, in all nine transfers from the original blood broth culture.

The result of these experiments demonstrates that the influenza bacillus grows well in the emulsion broth described. In this medium *B. influenzæ* was found living and capable of multiplying after 10 days at 37°C. (Table I).

Experiment 1 was repeated in emulsion broth prepared in the manner described from the mucoid growth of an organism classified as *Bacillus ozænæ*.

In this experiment the growth was washed off the surface of plain agar with 1 cc. of normal saline solution and the bacteria were killed by heating at 70°C. for 1 hour.   0.5 cc. of this sterile emulsion was then added to 5 cc. of plain broth. The emulsion broth was inoculated with 0.1 cc. of a blood broth culture of *B. influenzæ* after all blood cells had settled to the bottom of the tube.   24 hours later 0.1 cc. of this first emulsion broth culture was transferred to a second emulsion broth tube and simultaneously streaked on the surface of blood agar to determine growth.   This series of cultures was carried on successfully for ten transfers in emulsion broth, and then was voluntarily discontinued.   The ten consecutive cultures all showed typical colonies on the blood agar, and films of the last seven transfers showed typical bacilli and a few involution forms of *B. influenzæ*.

TABLE II.

Determination of Smallest Amount of Emulsion Capable of Stimulating Growth of B. influenzæ.

Tube No.	Amount of broth.	Amount of emulsion.	Growth.
	cc.	*cc.*	
1	5	0.5	+++
2	5	0.3	+++
3	5	0.1	+++
4	5	0.01	+
5	5	0	−

Experiment 2.—After it had been shown that an emulsion of heat-killed mucoid bacteria, when added to plain broth, is able to support growth of the influenza bacillus, it seemed desirable to learn how small an amount of the emulsion would suffice for this purpose.   Accordingly, dilutions of the emulsion in broth were made and inoculated with comparable amounts of a culture of influenza bacillus. After 24 hours incubation subcultures were made on blood agar to confirm growth in the cultures containing various dilutions of the emulsion.   These results are recorded in Table II.

0.01 cc. is evidently the lower limit of the growth-stimulating substance in this particular emulsion of heat-killed mucoid bacilli, since cultures containing this amount showed less growth than those in which larger quantities were used.

Experiment 3.—In order to determine whether the growth of *B. influenzæ* in the emulsion broth was more pronounced in the bottom of the tubes or at the surface of the broth, tubes were inoculated after all mucoid material had settled to the bottom. They were allowed to stand in the incubator for 24 hours and subcultures were made both from the thick residuum in the bottom of the tubes and from the superficial layers of the broth. The growth was compared with the following result.

Growth.

(a) From bottom of tubes.................................. +++

(b) From surface... +

The fact that this accessory substance appeared to be more concentrated in the immediate vicinity of the sedimented bacterial emulsion than in the upper portions of the culture fluid seems to indicate that the growth-stimulating factor is contained within the bacterial cell and slowly passes out into the surrounding fluid.

It therefore seemed reasonable to attempt to extract this substance from the bacterial emulsion. The addition of a clear bacterial extract to media, moreover, would have the advantage of making it possible to observe bacterial growth directly without the secondary transfer to blood agar. This was done in Experiment 4.

Experiment 4.—The growth of a mucoid organism was collected from agar plates and emulsified in plain broth, 1 cc. of broth being used to each plate. The emulsion was boiled for 5 minutes, and then centrifuged to separate the clear fluid extract from the bacterial bodies. This sterile extract was then tested for growth-stimulating action by the addition of decreasing amounts to plain broth. The medium prepared in this manner was inoculated with one drop from an emulsion broth culture (Table I, No. 6). The results of this experiment are recorded in Table III.

TABLE III.

Growth-Inducing Action of an Extract of Mucoid Bacteria on B. influenzæ.

Amount of extract.		Growth.
cc.	*per cent*	
0.3	6.0	++
0.1	2.0	++
0.05	1.0	+
0.01	0.2	−
0.001	0.02	−
0	0	−

It is evident from Table III that it is possible by simple boiling of an emulsion of mucoid organisms to obtain an extract which when added to plain broth is capable of inducing growth of *Bacillus influenzæ*. That the first extraction of the bacillary emulsion does not completely exhaust it of this growth factor is shown in the following experiment.

Experiment 5.—An emulsion of mucoid bacilli was made as previously described. A portion of this emulsion was heated at 60°C. for 1 hour and another portion was boiled for 5 minutes and then centrifuged and the clear supernatant extract pipetted off. The bacterial residuum was then washed in normal saline solution three successive times and the following experiment carried out.

	Growth.
5 cc. of plain broth + 0.5 cc. of unboiled emulsion heated to 60°C. for an hour......................................	+
5 cc. of plain broth + 0.5 cc. of extract from boiled emulsion...	+ +
5 cc. of plain broth + 0.5 cc. of residuum from boiled emulsion.	+ +
5 cc. of plain broth (control)................................	−

In this experiment the extract and the residuum from the boiled emulsion, when added to broth, gave even better growth than the emulsion heated at 60°C. for 1 hour.

That extraction of the growth-inducing substance is obtained simply by allowing the bacterial cells to remain in contact with broth for some time is shown in the following experiment.

Experiment 6.—To two tubes of plain broth there was added 0.5 cc. of a bacillary emulsion which had been heated to 60°C. for 1 hour. The tubes were then left in the ice box for 1 week. After this time, one tube was centrifuged and the clear supernatant fluid used as culture medium, while from the other, the supernatant fluid was pipetted off without being centrifuged. The two tubes were inoculated with 0.1 cc. of an emulsion broth culture of *B. influenzæ*. Good growth occurred in both tubes.

This experiment indicates that the growth-inducing substance passes from the bacterial cells into the surrounding fluid and there exists apart from the cell.

In the foregoing experiments it has been shown that it is possible to obtain good growth of *Bacillus influenzæ* in plain broth to which emulsions and extracts of mucoid bacteria have been added. It seemed reasonable, therefore, to seek the same growth factors in other microorganisms, and *Bacillus proteus* was selected as an organism which normally shows an abundant growth on ordinary media.

Experiment 7.—Agar plates were inoculated with *B. proteus*. After 24 hours growth, to each plate 1 cc. of normal saline solution was added, and the growth washed off and collected in a sterile centrifuge tube. The emulsion was boiled for 5 minutes. The sterile emulsion was then centrifuged and the supernatant fluid pipetted off. This extract was clear, yellowish in color and had a reaction of pH 7.6. 0.5 cc. quantities of this extract were used to enrich plain broth. After the addition of this amount the beef infusion broth remained clear, so that eventual growth could be indicated by the turbidity of the medium. Control cultures, however, were always made from the extract broth on blood agar or oleate hemoglobin agar, and in plain broth.

In the fluid medium thus prepared the following experiment was made. From a 24 hour culture of *B. influenzæ* in blood broth 0.1 cc. was transferred to *Proteus* Extract Broth 1; from this after 24 hours growth, to No. 2, and from this to No. 3. These three cultures all gave good growth and upon transfer to blood agar showed the typical colonies of *B. influenzæ*. Films also showed the typical small Gram-negative bacilli.

The experiment was voluntarily discontinued after the third transfer.

This experiment showed that *Bacillus influenzæ* would grow on a watery extract of *Bacillus proteus* for at least three generations. An experiment was next made with the whole bacterial emulsion of *proteus* in the same manner as described in Experiment 1. Here, however, growth of *Bacillus influenzæ* occurred only in the first two transfers after the blood broth culture. A considerable difference between the emulsions of the mucoid bacilli and the emulsion of *proteus*, therefore, seemed to exist. The explanation of this has not been found as yet, but it is reasonable to seek this in the morphological difference between these two microbes. The large capsule of the mucoid bacilli may be a better growth-inducing factor than the capsule-free *proteus*. The possibility that the capsule may contain some nutritional reserve for the bacillus has already been put forth by Toenniessen (8), who finds that the capsule consists of a polysaccharide, galactan, and that other bacteria grow better on the surface of cultures of Friedländer's bacillus than on plain agar.

Since it had been shown that it was possible to extract a growth-inducing factor from *Bacillus proteus*, tests were made to determine the influence of this extract upon the growth of *Bacillus influenzæ* in various sugar solutions (1 per cent of sugar in peptone water with Andrade indicator (9)).

Experiment 8.—To each 5 cc. of sugar medium was added 0.2 cc. of *proteus* extract. The tubes were then inoculated with 0.1 cc. of culture (No. 2 in the foregoing experiment) and incubated. The following sugars were used: lactose, mannitol, maltose, dextrose, saccharose, raffinose, inulin, and salicin. After 12 hours incubation a heavy clouding of the medium was visible in all tubes and the dextrose culture had turned slightly red. After 24 hours the culture containing dextrose was distinctly red, while those containing the other sugars remained colorless. At this point, control cultures on blood agar from all tubes showed pure growth of *B. influenzæ*. 4 days after inoculation the tubes showed the same reactions. On transfers to blood broth the cultures were all found to be living and pure.

Experiment 9.—It was considered of interest to determine whether or not the clear extract of *B. proteus* could be filtered through a Berkefeld filter without losing its potency. After the extract had been prepared as already described, it was passed through a Berkefeld filter N and the water-clear filtrate, after being proved sterile, was added to plain broth in the following amounts.

Broth.	Filtrate.	Growth.
cc.	*cc.*	
5	1.0	+++
5	0.5	+++
5	0.2	+
5	0.05	—
5	0.01	—
5	0	—

Growth was controlled by turbidity of the medium, by films, and by subcultures on blood agar and plain agar. These controls showed the growing organism to have the characters of *B. influenzæ*.

This experiment shows that the bacillary extract in question can pass through a Berkefeld filter without losing its growth-inducing property.

SUMMARY.

From the data presented in the foregoing experiments it is evident that *Bacillus influenzæ* will grow in a fluid medium consisting of plain broth to which have been added small amounts of emulsions or extracts of mucoid bacilli or of *Bacillus proteus*. The bacterial extracts may be made by simple boiling of the bacillary emulsions in broth or saline solution and centrifuging out the bacterial bodies; they may be filtered without losing their growth-inducing property.

Cultures of *Bacillus influenzæ* in bacterial extract broth, if not too small doses of the extracts were employed, always showed heavier growth than the control cultures in blood broth, and growth occurred at a considerably earlier period than in blood broth. In many instances growth could be seen after 3 to 4 hours, and a bacterial whirl was always visible after 6 hours incubation.

When the nature of the culture used for seeding is not stated, this was 0.1 cc. of the supernatant fluid of a blood broth culture.

All cultures were made in fluid medium; solid medium is much more difficult to use in connection with the extracts.

In explanation of the remarkable growth of *Bacillus influenzæ* in this blood-free medium, the idea is proposed that the growth-stimulating effect of the bacterial extracts is due possibly to substances of the same nature as the so called vitamines.

Further investigations on this principle of bacterial nutrition will appear in subsequent papers, together with a more thorough study of the sources and character of the growth-inducing substances.

CONCLUSIONS.

1. It is shown that *Bacillus influenzæ* will grow profusely in hemoglobin-free media consisting only of plain broth and emulsions or extracts of mucoid bacilli and *Bacillus proteus*.

2. The emulsions and the extracts can be boiled and filtered through Berkefeld filters without losing their growth-inducing property.

3. The growth-stimulating effect of the bacterial extracts is possibly due to substances belonging to the class of the so called vitamines.

BIBLIOGRAPHY.

1. Cantani, A., *Z. Hyg. u. Infectionskrankh.*, 1901, xxxvi, 29.
2. Neisser, M., *Deutsch. med. Woch.*, 1903, xxix, 462.
3. Grassberger, R., *Z. Hyg. u. Infectionskrankh.*, 1897, xxv, 453.
4. Luerssen, A., *Centr. Bakt., 1te Abt., Orig.*, 1904, xxxv, 434.
5. Ghon, A., and von Preyss, W., *Centr. Bakt., 1te Abt., Orig.*, 1904, xxxv, 531.
6. Putnam, J. J., and Gay, D. M., *J. Med. Research*, 1920, xlii, 1.
7. Thjötta, Th., and Eide, O. K., *J. Bact.*, 1920, v, 501.
8. Toenniessen, E., *Centr. Bakt., 1te Abt., Orig.*, 1920, lxxxv, 225.
9. Stillman, E. G., and Bourn, J. M., *J. Exp. Med.*, 1920, xxxii, 665.

STUDIES ON BACTERIAL NUTRITION.

II. GROWTH ACCESSORY SUBSTANCES IN THE CULTIVATION OF HEMOPHILIC BACILLI.

BY THEODOR THJÖTTA, M.D., AND O. T. AVERY, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

In a preceding paper it was shown by Thjötta (1) that *B. influenzæ* will grow on hemoglobin-free medium consisting of plain broth enriched by sterile suspensions or extracts of mucoid bacteria. It was suggested that under these conditions growth may be attributable in part to the presence in the bacterial extracts of substances belonging to the class of so called vitamines. It was found that the growth-promoting action of these substances of bacterial origin was not destroyed by boiling for 10 minutes or by passage through Berkefeld filters. In the present paper are presented the facts thus far acquired in the study of the growth requirements of the so called hemophilic bacilli: the growth-stimulating action of extracts of yeast and of vegetable cells, and the importance of blood as a source of growth accessory substances in the cultivation of bacteria.

The earliest reference in literature to the importance of accessory growth factors in the cultivation of microorganisms is made by Wildiers (2) (1901). This investigator observed that watery extracts of yeast cells greatly enhance the growth of yeast in a synthetic medium.

In confirming the observation of Pasteur on the growth of yeast in a medium composed of mineral salts and sugar, Wildiers found that the size of the inoculum was of the greatest importance; a small seeding would not suffice to initiate growth, while a larger inoculum grew abundantly in this medium. This fact suggested that the growth difference lay in some intracellular factor furnished by the heavier seeding which on the death and disintegration of certain of the yeast cells was liberated, and supplied to the surviving cells a necessary growth-inducing substance. This growth-stimulating principle in yeast to which Wildiers gave the name "bios" was contained within the cell and could be extracted by boiling yeast cells in water. The extract obtained in this manner and concentrated on a water bath was a yellowish, clear, syrup-like fluid. This intracellular substance, the so called "bios," was soluble in water and 80 per cent alcohol, and insoluble in concentrated alcohol and ether. It was resistant to acids but sensitive to alkalies. It could be filtered and dialyzed through paraffined paper.

The findings of Wildiers concerning "bios" were confirmed by Amand (3) who showed that this substance disappeared from the culture medium during growth. This observation Amand interpreted as evidence that "bios" was used up by the yeast cells during growth, and was not a product of their own metabolism.

The work of Wildiers and Amand seems to have been forgotten until quite recently when several investigators have adapted the principle of these earlier workers to methods for the measurement of the vitamine content of various substances. Bachmann (4) measures the vitamines in the test material by adding the sterilized substance to yeast cultures and measuring the amount of gas evolved as an index of growth acceleration; Eddy and Stevenson (5), in determining the presence of vitamine, compare the numerical increase of cells in cultures of yeast in a medium with and without the test substance. On the other hand, from their experiments on the growth of yeast in synthetic media without growth accessory substances, McDonald and McCollum (6) conclude that yeast must be capable either of growing without vitamines or of synthesizing these substances during growth.

While Wildiers and Amand distinctly pointed out the importance of extracts of yeast in the nutrition and growth of yeast cells, they did not extend their observations to the effect of these extracts upon the growth of other microorganisms, such as bacteria. It was not until after the discovery of the value of growth accessory substances in the nutrition of animals that the question of their significance in the growth of bacteria was considered. Bottomley (7) showed the existence in peat of certain substances that have a stimulating effect upon the growth of plants and soil bacteria. These substances he called "auximones" and considered them of the same importance in the growth of plants as vitamines in the nutrition of animals. These "auximones" can be extracted from peat in the same manner as the vitamines from plants, and this fact, together with their apparently uniform action seems to indicate the close relationship between these substances.

Following this discovery it was only a question of time when the principle of growth accessory substances should be brought into full use in the cultivation of bacteria.

In 1916, Lloyd (8) showed that the meningococcus required for its growth a rich supply of vitamines in the medium. Lloyd attributes the value of blood and serous fluids in the cultivation of meningococcus to their vitamine content and believes that Gordon's pea flour and Vedder's starch medium may also contain vitamine which may partly account for the value of these media. In explanation of the fact that meningococcus after artificial cultivation tends to grow with increasing readiness on ordinary media, this author suggests that in adaptation to a more or less saprophytic existence these organisms become independent of vitamine supply.

Cole and Lloyd (9) recommend as the optimum medium for gonococci one rich in amino-acids and vitamines, or "growth hormones." These authors

believe in the existence of two different "growth hormones," a substance present in blood cells and easily absorbed from the media by filtering, which seems to induce the initial growth, and a second substance found in tissues of plants and animals which has the power of inducing luxuriant secondary growth. Davis (10) pointed out that *B. influenzæ* requires in addition to hemoglobin a vitamine substance. He compared these two factors to the fat-soluble vitamine A and the water-soluble vitamine B. Davis showed that the vitamine supply could be obtained from plant and animal tissue. By placing sterile sections of vegetables on blood agar seeded with influenza bacilli, Davis demonstrated that better growth occurred around the pieces of vegetables than on the other portion of the plates, just as Grassberger many years ago called attention to the more luxuriant growth of Pfeiffer's bacillus around colonies of staphylococci.

Davis (10) also applied this principle in the cultivation of other bacteria. As sources of vitamines Davis added to media polished and unpolished rice flour, white and whole wheat flour. If these grains were allowed to sprout the growth was more profuse. The increasing growth on the media made from sprouting grain is explained as due to enzymotic activity in the sprouting process, resulting in changes in proteins, and inversion of starch into sugars.

In the preceding paper (1) on the growth of *Bacillus influenzæ* in broth containing sterile bacterial extracts, it was pointed out that this phenomenon may be explained in part at least by the presence of growth accessory factors, or vitamines, in the bacterial extracts. In this paper an attempt is made to analyze further the growth requirements of the so called hemophilic bacilli and to indicate the significance of growth accessory substances in bacterial nutrition.

EXPERIMENTAL.

Growth Accessory Substances in Yeast and Vegetable Cells.

Although extracts from bacteria of the mucoid variety may be readily prepared, their use is not practicable. In the present study extracts were prepared from yeast cells which are known to be rich in water-soluble vitamine, and which, as previously shown by Wildiers and Amand, have a marked stimulating effect upon the growth of yeast cultures. Extracts of fresh ripe tomatoes, green peas, and green beans were also used, since these vegetables are also valuable sources of growth accessory substances.

Preparation of Extract of Yeast.—100 gm. of brewers' yeast[1] were emulsified in 400 cc. of distilled water. Since the vitamines will stand boiling better in acid than in alkaline solution, the reaction of the suspension of yeast cells was adjusted to pH 4.6, boiled over a free flame for 10 minutes, and then allowed to sediment at room temperature. The clear supernatant extract was pipetted off and tested for sterility. The clear, sterile extract, unneutralized, was stored in the ice box and added to the medium immediately before use. The extract prepared in this manner was a clear, yellowish fluid. Chemical analysis showed the following nitrogen content:[2] total nitrogen, 0.116 per cent; ammonia nitrogen, 0.011 per cent; amino nitrogen, 0.039 per cent; peptide-bound nitrogen, 0.024 per cent; undetermined nitrogen, 0.042 per cent. Before addition of the yeast extract to media, the reaction may be readjusted to optimum for growth of *Bacillus influenzæ*, pH 7.3 to 7.5.

Preparation of Extract of Tomatoes.—Ripe tomatoes were treated in the following way: The skin surface was seared with a red hot knife and through this area a sterile fork was plunged. The tomato was then dipped in alcohol and flamed, then plunged into boiling water for a minute, the skin peeled off, and the stem removed with sterile forceps. The tomatoes were placed in a sterile enamel dish, and crushed with a sterile pestle. The reaction of the tomato juice in its natural condition was pH 4.2, and therefore required no readjustment before boiling, as in the case of the yeast emulsion. The crushed tomatoes were boiled for 10 minutes and the expressed juice either filtered through a Berkefeld filter (N) or cleared by centrifugation, stored at its original acidity, and the reaction readjusted before use. This extract was a perfectly clear, slightly yellowish fluid. Nitrogen partition on this particular extract was as follows: total nitrogen, 0.14 per cent; ammonia nitrogen, 0.014 per cent; amino nitrogen, 0.079 per cent; peptide-bound nitrogen, 0.00 per cent; undetermined nitrogen, 0.047 per cent.

---

[1] The yeast used in these experiments was supplied through the courtesy of Mr. F. Spitzner of the Central Brewing Company of New York.

[2] For the nitrogen determination on both the yeast and tomato extracts we are indebted to Miss Alma Hiller of the Hospital of The Rockefeller Institute.

Preparation of Extracts of Green Peas and Beans.—Fresh green peas were prepared by flaming the surface and opening the pods with sterile forceps and crushing the separate seeds out into a sterile dish. An equal amount of sterile distilled water by weight was added and the reaction of the emulsion adjusted to pH 4.6 After boiling for 10 minutes the extract was strained through glass wool and then filtered through a Berkefeld filter (N). The resulting extract was perfectly clear and yellowish in color. Similar extracts were prepared from string beans.

Stimulating Action of Vitamine-Like Substances on the Growth of Bacillus influenzæ.—Yeast and vegetable extracts prepared in the

TABLE I.

Stimulating Action of Growth Accessory Substances in Yeast and Vegetable Extracts on the Growth of B. influenzæ.

Dilution of extract in plain broth.*	Growth-stimulating action of.		
	Yeast extract.	Tomato extract.	Extract of peas.
1:10	++	++	++
1:100	++	++	++
1:1,000	+	−	−
1:10,000	−	−	−
Plain broth.	−	−	−

* The extract-containing broth was inoculated with 0.05 cc. of the supernatant fluid of a blood broth culture of *B. influenzæ.*

++ indicates good growth; + moderate growth; ± slight growth; − no growth.

manner described were tested for their stimulating action on bacterial growth by adding them in varying concentration to plain broth of pH 7.8. This medium was inoculated with 0.05 to 0.1 cc. of the supernatant fluid of a blood broth culture of *Bacillus influenzæ*. A bacterial whirl was often visible in the cultures seeded in this manner after 6 hours incubation, and was always marked after growth over night.

Titrations of the vegetable extracts were made in infusion broth to determine the lower limit of the growth-stimulating action (Table I). Table I shows that even in high dilutions extracts of yeast and

of fresh green vegetables are able to stimulate growth of *Bacillus influenzæ* in plain broth if seeded with a small inoculum from media containing blood.   When it is considered that the original extracts contain little nitrogenous matter (yeast extract 0.14 per cent) and that only one-thousandth of this amount is present in the dilution required for growth, it becomes obvious that the extracts do not serve merely as additional nutriment, but that their action is accessory in nature, similar perhaps to that of vitamines in animal nutrition. This resemblance is the more striking in that these extracts resist boiling for at least 10 minutes and are destroyed at autoclave temperatures (120°C. for 30 minutes) as shown in Table II.

TABLE II.

Relative Growth Capacity of Yeast and Tomato Extracts after Boiling and Autoclaving.

Temperature.	Tomato extract.	Yeast extract.	No extract.
	In plain broth, 1:10.*	In plain broth, 1:10.*	Plain broth.
10 min. at 100°C. 30 " " 120° "	++ −	++ −	− −

* Inoculated with 0.1 cc. of the supernatant fluid from an 18 hour blood broth culture of *B. influenzæ.*

In the literature the statement is frequently encountered that the growth value of culture media is greatly impaired by filtration.   This loss is attributed to absorption of the so called hormones during the process of filtering.   In order to test the effect on the growth-stimulating value of yeast extract after absorption with bone charcoal, the following experiment was carried out.

In each of two small flasks under sterile precautions 1 gm. of bone charcoal and 5 cc. of yeast extract (pH 5.4) were mixed.   One portion of the mixture was heated on a steam bath for 15 minutes in order to facilitate absorption; the other was placed at room temperature and frequently shaken.   At the end of the absorption period the charcoal was removed from suspension by centrifugation and the absorbed extract tested for its growth-inducing action.   Extract without charcoal was heated on a steam bath for 15 minutes to determine whether or not the additional heating affected its potency.   The results are recorded in Table III.

From Table III it is apparent that the growth-promoting power of yeast extract is susceptible to absorption by bone charcoal. It is, of course, obvious that the completeness of absorption is related to the concentration of the particular extract and the length of time allowed for absorption. It is evident that under the experimental conditions described the vitamine-like principle in yeast extract is absorbed by bone charcoal and that absorption occurs more promptly under the influence of heat.

From the data presented in the preceding experiments it is evident hat *Bacillus influenzæ* will grow when transferred by small inoculum rom blood media to plain broth containing extracts of yeast or vege-

TABLE III.

Effect of Absorption by Bone Charcoal on the Growth-Stimulating Action of Yeas Extract.

Dilution of extract in plain broth.*	Yeast extract absorbed.		Unabsorbed.	
	15 min. on steam bath.	2 hrs. at room temperature.	Unheated.	15 min. on steam bath.
1:10	±	+	++	++
1:20	−	±	++	++
1:50	−	−	++	+

* Inoculated with 0.05 cc. of the supernatant fluid of a blood broth culture of *B. influenzæ*.

table cells but fails to grow under similar conditions in the same broth without the addition of these extracts. However, for reasons to be discussed later, continued cultivation fails in broth containing only yeast or vegetable extracts.

While the chemical nature of these growth accessory substances is not known, they are analogous in behavior to the so called vitamines. Extracts containing these substances have been prepared from fresh vegetables and from bacterial and yeast cells. It has been found that they resist boiling for 10 minutes, that they are destroyed by autoclaving, that they contain but little available nitrogen, that they pass a Berkefeld filter with little or no impairment, but are absorbed from water solution by bone charcoal.

Growth Accessory Substances in Blood.

Blood has always been considered requisite for growth of Pfeiffer's bacillus, and the inability of certain organisms to multiply in the absence of blood or blood derivatives has constituted an absolute criterion for their differentiation as hemophilic bacteria. It is important at this point to emphasize again the fact previously mentioned that although *Bacillus influenzæ* grows luxuriantly when transplanted from blood medium to plain broth containing yeast extracts, cultivation cannot be continued for more than one or two transfers in yeast broth alone. Growth deficiency under these circumstances suggests that possibly some other substance may be carried over from the original blood culture in an amount sufficient to supplement the vitamine factor in yeast broth and that growth fails in succeeding cultures in this medium because this second substance is either exhausted by growth or lost by dilution on subsequent transfer. For purposes of discussion this second substance may be referred to as the "X" factor, and the vitamine-like substance as the "V" factor. The theoretical consideration of the presence of these two essential growth factors in blood requires for its substantiation the demonstration of the dual nature of the growth-stimulating property of blood. In the following experiments the interdependence of these two substances and their distribution in the various fractions of blood, body fluids, and crystalline hemoglobin will be discussed.

It has already been pointed out in the first part of this paper that one of the substances, the V factor, can be supplied from a source other than blood, as for instance, yeast. In explanation, therefore, of failure of continued growth of *Bacillus influenzæ* in yeast broth alone, it is suggested that the X substance lacking in this medium is supplied in the first instance by the inoculum from the original blood broth, and that the amount of X furnished in this way is sufficient when supplemented by an excess of the V factor from yeast to sustain growth in the first transfer, but that in subsequent cultures the X factor is quickly lost or perhaps used up by growth of the bacilli. That this second substance, the so called X factor, is actually carried over from blood broth with the first inoculum is shown in the following experiment.

The red blood cells were sedimented from a blood broth culture of *B. influenzæ* by slow centrifugation; the supernatant culture fluid was again centrifuged and the sedimented bacteria were washed three times in large volumes of sterile salt solution to remove any trace of X substance adherent to them. The washed bacilli were resuspended in salt solution to the volume of the original culture and the relative growth capacity of the washed and unwashed bacilli from blood broth was tested in plain bouillon containing yeast extract in 10 per cent concentration.

The facts recorded in Table IV and substantiated in repeated experiments justify the assumption previously made that in addition to the V substance in blood which finds its analogue in yeast extract, there is also present another substance (X) equally essential to growth

TABLE IV.

Relative Growth Capacity of Washed and Unwashed Influenza Bacilli from Blood Culture in Yeast Extract Broth.

Inoculum.	10 per cent yeast extract broth (V) inoculated with.	
	Washed bacilli from supernatant fluid of blood broth culture (no X).	Unwashed bacilli from supernatant fluid of blood broth culture (X present).
cc.		
0.1	−	++
0.05	−	++
1 loop.	−	−

Controls: 0.1 cc. of supernatant blood broth in plain broth without yeast extract showed no growth. 1 loop of washed bacteria on blood agar yielded good growth.

of *Bacillus influenzæ.* This accessory X substance, to the lack of which in media hemophilic bacteria are peculiarly sensitive, is capable in extraordinarily small quantities, such for instance as may be carried over in a single inoculum from blood broth, of supplying the necessary growth conditions. Although this second substance, the so called X factor, can function in minute amounts, it is unable by itself to induce growth, for, as shown by the controls in Table IV, the same inoculum which yields growth in broth containing yeast extract fails to grow in plain broth alone, although in both instances the amount of X carried over in the inoculum is the same.

These two substances may be further differentiated by their relative susceptibility to heat. It is known that the clear fluid extracted from the coagulum of whole blood by short exposure to the temperature of boiling water supports growth of *Bacillus influenzæ*. This blood extract, therefore, presumably contains both the X and the V substances, and its addition to plain media in small amounts suffices for growth of hemophilic bacilli. Since, by boiling for several minutes, substances can be extracted from blood which still possess the growth-stimulating properties exhibited by unheated blood, it is evident that the factors concerned in promoting growth are not destroyed by

TABLE V.

Effect of Heat on the Growth Accessory Substances of Blood.

Dilution of blood extract in plain broth.	Blood extract.* 10 min. at 100°C.		Blood extract.* Autoclaved at 120°C. for 30 min.	
	Without yeast extract.	With yeast extract, 0.5 cc. (V).	Without yeast extract.	With yeast extract, 0.5 cc. (V).
1:10	++	++	−	++
1:100	++	++	−	++
1:1,000	−	+	−	+
1:10,000	−	−	−	−
1:100,000	−	−	−	−

* Prepared by placing a tube containing rabbit blood in boiling water for 10 minutes and using the clear extracted fluid after removal of coagulated material by centrifugation.

short exposure to this temperature. In studying the effect of heat on the growth-stimulating action of tomato and yeast extracts (Table II) it was found that boiling for at least 10 minutes did not appreciably impair their action, while exposure in the autoclave to 120°C. for 30 minutes greatly diminished or completely destroyed their potency. If blood, therefore, contains a substance analogous in its behavior to the vitamine-like principle extracted from yeast, then blood which has been autoclaved should fail to support growth of *Bacillus influenzæ*, since the V factor would be destroyed under these conditions. On the other hand, if the X factor of blood is stable to heat, then the autoclaved blood should be reactivated by the

addition of the fresh V substance in yeast extract. To test the validity of this assumption the experiment presented in Table V was carried out.

From Table V it is evident that the accessory substances in blood essential to growth of hemophilic bacilli are not destroyed by exposure to 100°C. for 10 minutes, since extracts prepared from boiled blood are active in promoting growth. It is further apparent that these same blood extracts after autoclaving for 30 minutes at 120°C. are no longer capable by themselves of supporting life of the bacilli. The experiment further demonstrates the interesting fact that the substance or substances in blood which are destroyed by excessive heat can be replaced by the addition of the growth-stimulating principle extractable from yeast. An analysis of these observations on the heat sensitiveness of the growth-promoting substances in blood indicates clearly that differences exist in thermostability on the basis of which it is possible to separate these two factors. The X factor, so called, is heat-stable and unaffected in action by exposure to steam under pressure at 120°C. for $\frac{1}{2}$ hour. On the other hand, the so called V factor of blood, like the similarly reacting substance in extracts of yeast, is relatively more labile and is destroyed at the autoclave temperature.

Before passing to the question of the distribution of these growth accessory substances in the various fractions of blood, it is well to emphasize the peculiar sensitiveness of the hemophilic bacilli to a lack of either of these two factors in media, and the relative differences in the effective amounts of each. It has been pointed out (Table IV) that in a small inoculum from blood media, a sufficient amount of the X substance may be carried over to cause growth in blood-free broth, providing yeast extract, which supplies the V factor, is also added. In other words, mere traces of the X substance in the presence of this vitamine-like principle suffice for growth, but neither separately can function even when present in excess. This dual action, and the relative amounts of each factor necessary, particularly the minimal effective dose of X, are important in the technique of determining the presence or absence of either factor, since it is possible to carry over with the inoculum enough of the particular factor lacking in a test medium to permit growth. The nature of the seeding, therefore, must

be carefully chosen so that the culture from which it is taken shall contain only a minimum of the particular factor sought for in any given medium or extract. For example, when testing for the presence of the V factor in a given extract or medium, the seeding should be made from the supernatant fluid of a blood broth culture, since in this instance an effective amount of the X substance is carried over with little or no V factor. On the other hand, in demonstrating the presence of the X substance in blood or a derivative of blood, the medium in question should be inoculated from a yeast extract broth culture derived as above, since under these conditions a minimal and ineffective amount of X is transferred in the inoculum. In testing for the X substance an excess of the V factor should always be added in the form of yeast extract, or its equivalent.

Distribution of the Growth Accessory Substances in Blood and Blood Derivatives.—A comparative study of the distribution of these growth accessory factors in blood and blood derivatives was made by determining the presence or absence of growth of *Bacillus influenzæ* in plain broth enriched with graduated amounts of ascitic fluid, serum, blood extract, and solutions of laked red blood cells and of crystalline hemoglobin. The sterile ascitic fluid, untinged with hemoglobin, was obtained from a patient suffering from cirrhosis of the liver and had been stored without antiseptic in the ice box for several months prior to use. The serum, blood extract, and solution of laked blood cells were prepared from freshly drawn defibrinated rabbit blood. The serum was separated from sterile defibrinated blood by repeated centrifugation, care being taken to obtain a specimen with no visible traces of blood pigment. The blood extract consisted of that fraction of whole blood expressed from the coagulum after boiling for 10 minutes, and separated from the coagulated proteins by prolonged centrifugation.

The solution of laked red blood cells was made as follows: The red cells from 20 cc. of sterile defibrinated blood were removed by centrifugation, washed three times in sterile salt solution (50 cc. each time), and taken up in sterile distilled water to the original volume of blood. After laking, the cell residue was sedimented by centrifuging and the clear supernatant fluid pipetted off and used as hemoglobin solution. By gasometric analysis 1 cc. of this solution contained 0.1 gm. of

hemoglobin. The crystalline hemoglobin[3] was prepared by the method of Welker and Williamson (11) from ox blood. The crystalline hemoglobin, as is usual with dry preparations, had lost its oxygen-carrying capacity. A water solution of the crystals (10 per cent by weight) was rendered sterile by passage through a Berkefeld filter (N).

TABLE VI.

Distribution of the Growth Accessory Substances V and X in Blood and Blood Derivatives.

Dilution in plain broth.*	Ascitic fluid.		Serum.		Blood extract.		Solution of laked red cells.†		Solution of crystalline hemoglobin.‡		Yeast extract.
	Without yeast extract.	With yeast extract.	Without yeast extract.	With yeast extract.	Without yeast extract.	With yeast extract.	Without yeast extract.	With yeast extract.	Without yeast extract.	With yeast extract.	10 per cent as control.
1:2	−	++									
1:5	−	++									
1:10	−	−	−	++	++	++	++	++	−	++	−
1:100	−	−	−	+	++	++	++	++	−	++	
1:1,000			−	+	−	+	+	++	−	++	
1:10,000			−	−	−	−	−	++		++	
1:100,000			−	−				+§		++	
1:200,000										+‖	

* All tubes inoculated with 0.05 cc. of an 18 hour yeast extract broth culture of *B. influenzæ*.

† Contained 10 gm. of hemoglobin per 100 cc.

‡ Contained 10 per cent crystalline hemoglobin.

§ Represents a final concentration of 1:1,000,000 hemoglobin.

‖ Represents a final concentration of 1:2,000,000 crystalline hemoglobin by weight.

The ascitic fluid, serum, and blood derivatives prepared as described above were added to plain infusion broth in the dilutions indicated and inoculated with 0.05 cc. of an 18 hour yeast extract broth culture of *Bacillus influenzæ*. Growth was controlled by subculture on blood agar and by second transfer to yeast extract broth alone.

[3] We are indebted to Dr. J. P. Peters, Jr., of the Hospital of The Rockefeller Institute for the preparation of crystalline hemoglobin used in these experiments.

An analysis of Table VI reveals certain facts concerning the distribution of the growth accessory factors in blood and blood derivatives. In the first place, it is evident that *Bacillus influenzæ* will not grow in serum in concentration as high as 10 per cent in broth or in ascitic fluid diluted with equal volumes of broth. In the presence of yeast extract, however, growth occurred under otherwise identical conditions in ascitic fluid broth 1:5 and in serum broth 1:1,000. Secondly, blood extract and hemoglobin solution freshly prepared from laked red cells in broth were able to stimulate growth in dilutions as high as 1:100 and 1:1,000 respectively. The addition of yeast extract to broth containing these blood derivatives permitted growth in even higher dilutions; namely, 1:1,000 of blood extract, and 1:100,000 of the solution of laked cells. Finally, the addition of a solution of crystalline hemoglobin to broth in concentrations equivalent to the solution of laked red cells failed to yield growth, while in dilutions equivalent to 1:200,000 of laked cells the same solution of crystalline substance afforded excellent growth when yeast extract was added. It should be noted that for purposes of comparison the concentrations are expressed in Table VI in terms of dilution of the original body fluids or their equivalents. However, since in the solutions of laked cells and of crystalline hemoglobin the content of this substance by weight is 10 per cent, the actual dilution of hemoglobin sufficing for growth is, therefore, ten times greater than that recorded in the protocol. In terms of actual amount of crystalline hemoglobin by weight, these figures represent concentrations of 1:100 and 1:2,000,000 respectively.

In attempting to determine the distribution in blood of the so called growth accessory factors V and X, it must be borne in mind that the methods employed are not strictly comparable and that the results are merely relative and of necessity must vary with each individual specimen of serum or blood. Nevertheless, the growth differences are sufficiently great to make it apparent that the greatest concentration of both these substances is associated with the cell fraction of the blood, and that serum and ascitic fluid, on the other hand, contain little or no measurable quantity of the vitamine-like principle and only relatively small amounts of the X substance, as is evident from the fact that by themselves they are separately incapable of support-

ing growth. The quantity of X substance in pure serum is relatively slight as compared with its abundance in solutions of laked cells and crystals of hemoglobin. This fact makes it seem not unlikely that the red blood cell is the source of this substance and that the presence of X in serum or ascitic fluid is purely accidental, due to conditions which permit its escape from the blood cells. The activity of this substance in crystalline hemoglobin in dilutions as great as 1:2,000,000 by weight is strong evidence that it is some constituent of the red blood cell which may function as a catalytic agent. It must be borne in mind that the detection of the X substance by itself in solutions of crystalline hemoglobin, in ascitic fluid, and in serum is made possible only when these are used in combination with vitamine-containing extracts from yeast or from other extraneous sources, since the X substance alone is inactive and unable to support growth without the complementing V factor.

In attempting to interpret the facts brought out in Table VI, it is of further interest to observe the distribution and relative concentration of V factor, or vitamine-like principle, in the various fractions of blood and blood derivatives. Just as the X substance appears to be intimately associated with the cell fraction of blood, so the V factor is apparently found in greatest concentration in the blood corpuscles. Because of the greater susceptibility of the V factor to heat and chemical manipulation, and owing to the fact that it is present in blood in lower concentration than the X substance, this vitamine-like principle is active only when the intact red cell and solutions or extracts of these cells have not been subjected to untoward conditions. For example, the chemical procedures incident to the preparation of crystalline hemoglobin cause complete loss of this factor, while the more stable X substance remains unimpaired. Like the V factor, extractable from yeast and vegetable cells, the corresponding substance in blood extracts withstands boiling for at least 10 minutes, but is destroyed by exposure to 120°C. for 30 minutes, neither of which procedures, however, interferes with the peculiar properties of the X substance in blood. In the specimen of blood extract used in this experiment, the V factor exerted its growth-stimulating action in dilution of 1:100, and in the unheated solution of laked cells, in which the hemoglobin was physiologically active as shown by its oxygen-

carrying capacity, the V factor was present in dilutions as high as 1:10,000. On the other hand, as already noted, solutions of crystalline hemoglobin were devoid of vitamine-like property and required the addition of yeast extract to supplement the X substance present.

<div align="center">DISCUSSION.</div>

As already pointed out, the importance of growth accessory substances in the cultivation of bacteria has been appreciated by numerous observers, and in the case of the hemophilic bacilli has been emphasized particularly by Davis. The foregoing experimental data are presented, therefore, not merely to direct attention to the relation of these substances to the growth of *Bacillus influenzæ*, but rather with the hope that as these studies progress, they may furnish the basis for a more accurate understanding of bacterial nutrition, and that the principles involved may find wider application in the cultivation of organisms other than those of the hemophilic group.

In preceding papers (1, 12) it has been shown that the substances requisite for growth of *Bacillus influenzæ* can be supplied from a source other than blood. The mucoid material elaborated during growth of certain bacteria, together with the dead bodies of these organisms in hemoglobin-free broth, has been found to furnish the accessory substances necessary for the cultivation of the hemophilic bacilli.

The present paper concerns itself with an attempt to analyze the accessory factors in blood which have to do with the peculiar nutritional requirements of the hemophilic bacteria. It is shown that the growth accessory substances in blood involve two distinct and separable factors, neither of which alone suffices to stimulate growth. One of these factors is analogous in its behavior to substances belonging to the class of so called vitamines, and because of this similarity is referred to in the text as the V factor. The other factor is less easily defined and is spoken of as the X substance. On the basis of relative differences in susceptibility to heat, these two factors may be separated one from the other. The vitamine-like principle in blood is destroyed by exposure to a temperature of 120°C. in the autoclave, while the X substance resists heating under these conditions. These substances, however, remain unimpaired in blood sub-

jected to the temperature of boiling water for 10 minutes. Extracts expressed from the coagulum of boiled blood contain both these factors and are capable, therefore, of supporting growth of *Bacillus influenzæ*. On the other hand, autoclaved blood contains only the more stable X substance and is incapable by itself of stimulating growth unless reactivated by the addition of the V factor from another source. That the vitamine-like substance in blood can be supplied from other sources is shown by the fact that extracts of yeast, tomatoes, green peas, and beans possess the property of reactivating an otherwise inert medium containing only the X substance.

Furthermore, it is shown that the addition to plain broth of active extracts of yeast or vegetable cells in dilutions as high as 1:1,000 suffices to initiate growth of *Bacillus influenzæ* when seeded from blood media. However, the fact that cultivation cannot be continued for more than one or two transfers in yeast extract broth alone suggests that in the first instance some of the X substance is carried over in the inoculum from the original blood culture in an amount sufficient to supplement the yeast broth and that growth fails in succeeding cultures because this X factor is either exhausted by growth or lost by dilution on subsequent transfer.

Moreover, study of the relative distribution of these two factors in the constituents of blood demonstrates the fact that they are present in greater concentration in the cellular elements than in the serum or plasma. The facts so far acquired indicate that the red blood cell is the carrier of the vitamine-like principle and that this substance, like its analogue in yeast cells, is intracellular in nature and can be extracted by the methods described. While the chemical nature of this substance is not known, it is presumably analogous to the so called vitamines. It has been found that similar growth-accelerating substances capable of replacing the V factor in blood can be extracted from bacterial and yeast cells and from fresh vegetables. These extracts contain but little nitrogen, they are destroyed by autoclaving, they are water-soluble, they pass a Berkefeld filter, but are absorbed readily from heated solutions by bone charcoal.

The experimental data recorded above indicate that in blood at least the X substance is intimately associated with or a derivative of hemoglobin. The fact that the X factor in crystalline hemoglobin

can function in dilutions as high as 1:2,000,000, when supplemented by an excess of the V factor from yeast extract, suggests that it may act as a catalytic agent.   Further observations on the nature of the X substance in blood and its presence in material other than animal tissue will be presented in a subsequent paper.

CONCLUSIONS.

The hemophilic bacteria of which *Bacillus influenzæ* serves as a type require for their growth two distinct and separable substances, both of which are present in blood and neither of which alone suffices. These substances are (*a*) a vitamine-like substance which can be extracted from red blood corpuscles, from yeast, and from vegetable cells, which is relatively heat-labile and absorbed from solution by certain agents; (*b*) a so called X substance which is present in red blood cells, is heat-stable, and acts in minute amounts.

BIBLIOGRAPHY.

1. Thjötta, Th., *J. Exp. Med.*, 1921, xxxiii, 763.
2. Wildiers, E., *La Cellule*, 1901, xviii, 313.
3. Amand, A., *La Cellule*, 1904, xxi, 329.
4. Bachmann, F. M., *J. Biol. Chem.*, 1919, xxxix, 235.
5. Eddy, W. H., and Stevenson, H. C., *Proc. Soc. Exp. Biol. and Med.*, 1919–20, xvii, 122.
6. McDonald, M. B., and McCollum, E. V., *J. Biol. Chem.*, 1921, xlv, 307.
7. Bottomley, W. B., *Proc. Roy. Soc. London, Series B*, 1915–17, lxxxix, 102, 481.
8. Lloyd, D. J., *J. Path. and Bact.*, 1916–17, xxi, 113; *Brit. Med. J.*, 1916, ii, 143; 1917, i, 11.
9. Cole, S. W., and Lloyd, D. J., *J. Path. and Bact.*, 1917, xxi, 267.
10. Davis, D. J., *J. Infect. Dis.*, 1907, iv, 73; 1917, xxi, 392.
11. Welker, W. H., and Williamson, C. S., *J. Biol. Chem.*, 1920, xli, 75.
12. Thjötta, Th., and Avery, O. T., *Proc. Soc. Exp. Biol. and Med.*, 1921, xviii, 197.

THE ETIOLOGICAL RELATION OF BACILLUS ACTINOIDES TO BRONCHOPNEUMONIA IN CALVES.

BY THEOBALD SMITH, M.D.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

PLATES 42 TO 50.

In the early months of 1917 an outbreak of pneumonia occurred among calves belonging to a large dairy herd in which about 100 calves are raised annually. Up to May 1, eleven cases had been autopsied. Nine of these were killed in advanced stages of the disease and two died. Scattering cases occurred at the rate of about one a month into November. Of these, one died and seven were killed. In the course of the investigations a minute bacillus was isolated whose etiological relation to the pneumonia was left in doubt.[1] In this outbreak a peculiar disease of the kidneys occurred in ten out of the nineteen cases. The lesions were situated in the cortex and consisted of foci of sclerosis with destruction of the secreting tissue.

More than 2 years later, in October, 1919, there began in the young stock of the same herd a similar series of cases. Up to March of 1920, ten cases came to autopsy. Of these four had died and six were killed. The kidney lesions were absent. A belated case was observed in June. There may have been more cases which recovered without being detected, since no careful clinical examination was made and the. disease was recognized only when the respiration had become sufficiently abnormal to warrant removal from the herd. The ratio of animals openly diseased to the entire number of calves was relatively low. This may have been due to the fact that the calves were kept in groups of six to eight in adjoining open pens, those of nearly the same age being kept together.

---

[1] Smith, T., *J. Exp. Med.*, 1918, xxviii, 333

In the second outbreak the lesions of the respiratory tract, more particularly of the lungs, were the chief if not the only ones. In the first, as stated above, a nephritis was associated with over half the cases. In the present article references to the nephritis will be omitted and made the subject of a separate paper. A description of the bacillus was published in 1918.[1] Its chief distinguishing characters are briefly as follows:

1. Multiplication in the form of small flakes up to 1 mm. in diameter which consist of parallel bundles of filaments, each filament terminating in two club-like expansions, one at each end. The flake thereby becomes more or less rounded, mulberry-like, and resembles a sphere with clubs projecting from the central mass. In this *Actinomyces*-like growth and within the sheathing filaments are chains of minute bacilli.

2. The sheaths and clubs are produced in the condensation water of coagulated serum and to a less extent on the sloped surface but not on agar plus tissue or blood. On these latter media the growth is feeble and the organism appears in the form of rods without the sheaths. It often fails entirely.

3. On ordinary media such as plain agar and bouillon the bacillus multiplies very faintly or not at all.

4. After 10 or more days growth on the agar plus blood or tissue, minute refringent bodies are found free in large numbers and also within the remaining rods. In some cultures all bacilli had disappeared. This phenomenon has been tentatively interpreted as spore formation.

5. The bacillus fails to produce any appreciable lesions in small animals.

Pathological Anatomy and Bacteriology of Individual Cases.

The gross appearances of the lungs differed very much from animal to animal but the cases could be grouped according to certain characters into acute, chronic, and intermediate types. The important features of the pneumonia, both pathological and bacteriological, are best presented by a brief description of certain cases which will form the basis for a discussion of the etiology. For the names applied to the different lobes of the cow's lungs in these pages, the reader is referred to the description of Text-figs. 1 and 2, page 450.

No. 440.—Female calf, aged 49 days. Died suddenly Nov. 12, 1919, and was autopsied within a few hours. There is no history of any pronounced illness preceding death, although this may have been overlooked by the attendant.

The lesions are restricted to the respiratory organs. The lungs are affected symmetrically. Both cephalic and ventral lobes are solidified with the exception

of six or more small air-containing regions along caudal border of right cephalic lobe and several along dorsal margin of the same lobe. Near root of right ventral lobe there is an air-containing region several centimeters in diameter. The large caudal lobes are solidified in the cephalic third or half. The involved tissue covers much more of the diaphragmatic than of the dorsal surface of these lobes. Air-containing lobules encroach on the hepatized territories and foci of collapsed lobules extend into and permeate the air-containing portions. The small azygous lobe consists of inflated and dark red collapsed regions intermingled.

The involved lobes are as firm as liver tissue to the touch. Their dimensions are about twice those of lungs normally collapsed. The gross appearance of the pneumonic regions differs from lobe to lobe. The cephalic and ventral lobes show closely set, grayish dots 1 to 3 mm. in diameter on a dark red ground, excepting a strip comprising the dorsal border, where the tissue is uniformly dark red, lacking the lighter dots. Similarly, the caudal lobes show the mottled character in a strip adjacent to the ventrals and the smooth, more uniform condition caudally, bordering on the still air-containing regions of these lobes. The mottling is also found on section. Embedded in one ventral lobe there are several foci, 4 to 5 mm. in diameter, which are made up of a semisolid caseous matter. Quite small foci 1 to 2 mm. in diameter of similar character are found here and there in the other lobes.

Larynx and trachea are normal. At bifurcation there is a mass of whitish mucoid substance. Both bronchi are deeply congested and flecks of whitish, soft, mucoid material are scattered over the mucosa. When the lobes are cut across and gently compressed, minute whitish molds are forced out of the smaller bronchioles. The lymph nodes, both mediastinal and bronchial, are somewhat enlarged, on section slightly congested and juicy. Occasional whitish, point-like foci are seen on the cut surface. The pleura is normal. The heart is flabby; pericardium normal. In the fat around the base of the right ventricle there is some patchy hemorrhage. Both sides of the heart contain large, dark, soft clots extending as cylindrical molds into all the large vessels.

Sections from fixed and hardened tissue of the various lobes stained in eosin and methylene blue present the following features. The small air tubes are filled with a mixture of cells and a peculiar, faintly bluish red, homogeneous material, probably mucus. The relative amount of this and of cellular material varies from tube to tube. Within the parenchyma there are foci consisting each of a group of alveoli which are packed with cell masses and the homogeneous substance referred to above. Among the desquamated alveolar epithelial cells are smaller cells the nuclei of which have contracted into irregular star-shaped, solidly stained masses of chromatin or into other irregular shapes, simulating the nuclei of polynuclear leucocytes. They are either lymphocytes or endothelial cells. A few polynuclear leucocytes permeate the mass of cells and debris. The cell foci may be so numerous as to coalesce, or there may be between them a zone of alveoli in which only a few desquamated alveolar cells are seen. It is these cell foci which appear to the naked eye as the grayish subpleural dots. The cell masses in the bronchioles and the alveoli are the same in character.

Another feature of significance is the presence within the smallest air tubes of what appear to be ingrowths from the wall (Figs. 1 to 3). These ingrowths consist of cells of the types described as present in the alveoli, embedded in a feebly stained matrix. The epithelium is missing where the ingrowth takes place. Within this matrix are dense masses of bodies varying in shape from minute rods in small colonies to groups of roundish bodies 2 microns in diameter with only the outline stained (Figs. 4 to 7). These latter bodies are also found in the alveolar cell masses but much less abundantly than colonies of minute bacilli (Figs. 8 and 9).

The origin of the cloud-like masses of bacteria pushing into the lumen of the air tube is obscure. A prolonged examination of sections from this and other cases makes it probable that the bacteria start in the cytoplasm of the epithelial cells, develop into colonies, and thereby cause a great increase in the size of the cells and their eventual destruction. The mass thus projects into the lumen where some of the migrating cells fuse with it, producing the characteristic obstructing plug. It is doubtful whether there is any actual hyperplasia of the epithelial cells. In the cell foci of the parenchyma a similar invasion of the alveolar cells takes place, leading to destruction of such cells and the appearance of hemorrhages and of the lymphocyte cell masses.

Cultures were made on slanted coagulated horse serum and slanted agar with and without a few drops of horse blood. All tubes were hermetically sealed and incubated. The cultures were made both by transferring bits of lung tissue and by inoculating the tubes with a heavy platinum wire thrust into the pneumonic tissue. After several days, growth appeared and in all but a few tubes *B. actinoides* was present in pure culture. In the remaining tubes a few foreign colonies were also present.

No. 450.—Black and white female calf. Born Oct. 27, 1919. Attendant reported that on Nov. 27 the calf did not eat its food and that breathing was rapid. From that day on the temperature was taken several times and found to be around 40.5°C. There was much coughing, the respirations continued short and superficial, and emaciation progressive. Dec. 4. The calf, then weighing 85 pounds, was killed by stunning with a heavy blow, clamping the trachea to prevent aspiration of stomach contents into the lungs, and opening the vessels of the neck. There was nothing noteworthy about the organs excepting the respiratory tract.

When the thorax was opened the lungs collapsed with force. The distribution of the pneumonic lesions is somewhat less extensive than in the preceding case. Both cephalics and ventrals are entirely consolidated. The cephalic half of the azygous lobe and a narrow strip along the cephalic margin of the right caudal lobe are involved. The left caudal lobe is intact. There is a narrow margin of air-containing lung tissue along the dorsal ridge of both lungs almost to the cephalic tip, also small inflated lobules in the left ventrocephalic lobe.

The condition of the consolidated lobes is not the same throughout. The right cephalic and the right ventral lobes are much larger than the normal collapsed

condition would be. They are grayish red to grayish yellow in appearance. The remainder of the affected lung is but a trifle larger than in the normal collapsed state and dark red with a faint, regular mottling of a lighter color. In this lung the grayish dots or granulations characteristic of the preceding case are absent. The pleura is normal.

The lower portion of the trachea and the main bronchi at the bifurcation contain masses of a viscid, opaque, whitish, dough-like mucopus. The same glairy, pearly white masses can be expressed from the cut ends of the minute air tubes of the dark red lobes. Only a small amount is expressible from the air tubes of the large grayish lobes. This glairy matter is very tenacious and breaks up into fragments when crushed between cover-glasses. The bulk of the material is made up of polynuclear leucocytes. The dark reddish tissue is quite moist when rubbed on covers, the grayish less so. The latter leaves on the cover-slip a smeary, milky film. Projecting from the right ventral lobe is a whitish mass the size of a pea. It is made up of a thin walled cyst filled with a readily dislodged, dough-like mass. Cover-glass films show abundant alveolar epithelium, polynuclear leucocytes, and several dense masses of very fine, rather feebly stained bacilli. Films from tracheal and bronchial mucopus and from lung parenchyma contain the same cellular and bacterial elements.

The difference between the gross pictures of the lungs of Nos. 440 and 450 is paralleled by differences in the microscopic picture. The peculiar cell infiltration in No. 440 has disappeared and polynuclear leucocytes have taken its place. The cell plugs in the air tubes contain chiefly polynuclears among which are scattering alveolar cells. The peculiar amorphous, homogeneous material which was regularly associated with colonies of bacteria in No. 440 is absent and bacteria are not detected. The parenchyma is the seat of polynuclear infiltration in foci which coalesce in the older stages and in the more recent stages gather irregularly in the collapsed tissue. The invasion of new territories is characterized by a ring of fibrin-blocked alveoli around the invaded air tube and occasionally hemorrhages. The interlobular tissue contains oval masses of fibrin lodged in the distended lymph spaces.

The difference between the two cases is further indicated by a new feature. All small air tubes are surrounded by a loose zone of cells which resemble plasma cells in form and the basophilic character of the cytoplasm and which collect in the subepithelial tissue.

Cultures prepared from the different lobes by transferring bits of lung tissue to horse serum, agar and blood, and plain agar, as well as by inoculating the same kind of media with heavy and fine platinum wire forced into the lung tissue yielded cultures of *B. actinoides* which were pure with the exception of those from the azygous lobe in which a few large streptothrix-like colonies appeared. The tubes inoculated with the heavy platinum wire developed, whereas most of those inoculated with a fine wire remained sterile.

No. 446.—This is a case which duplicates in all particulars No. 450 (Fig. 14). This calf was born in September and was 2 months and 2 days old when killed.

Cultures prepared from the different lobes in the manner described for No. 440 developed, with few exceptions, growths of *B. actinoides*. A few stray colonies of other kinds were present in several tubes.

No. 432.—This case may be classed as intermediate between the first and the following group. Female, born June 21; killed Oct. 21, when 4 months old. The meager data indicate that it was sick with some respiratory affection late in July and early in August. Early in October the respiration became short and quick, there was frequent coughing, and the calf breathed with mouth open. The respirations continued short and labored until it was killed. The temperature fluctuated between 39° and 40°C. The animal was killed by a stunning blow on the head, clamping trachea, and severing vessels of neck.

The consolidation of the lungs is of the usual extent. The pneumonic process involving completely the cephalic half of both lungs has invaded both caudal lobes and is separated from the air-containing tissue by a sharp, irregular, jagged line. Pneumonic lobules also appear as islands in these lobes. The trachea contains whitish, mucoid masses and the small air tubes of the affected lobes are filled with molds of similar material.

The hepatization is very firm, the affected lobes about twice the dimensions of the normal collapsed state. The surface is mottled like that in No. 440. Necrosis is absent. The cut surface shows the walls of the small air tubes distinctly thickened. In general the gross appearance indicates that the process is more or less of the same age throughout, except in the caudal lobes, where it is freshest. The mucopurulent molds from the small air tubes and the loose masses in the trachea contain large numbers of very minute and somewhat larger bacilli. Histological examination of sections from the different lobes indicates a transition in the pathological process from that of No. 440 to that of No. 450. In some regions the infiltrating cell masses are mononuclear, the contents of the minute air tubes largely made up of the homogeneous, bluish stained substance and groups of minute bacilli. In others, polynuclears predominate both in the parenchyma and the air tubes, and bacteria are rare or absent.

The bacteriology of the processes in this lung is complicated. On the agar and blood agar slants many roundish, rather fleshy colonies develop which have the cultural characters of the hemorrhagic septicemia group but with very low virulence. The general presence of *B. pyogenes* is indicated by liquefying colonies in all horse serum tubes. A third type of bacilli not identified was present in some tubes. Identifiable by its peculiar growth, *B. actinoides* was demonstrated as occurring in most tubes, but repeated attempts to obtain it in pure culture failed, owing to the predominance of the other types.

A type of pneumonia more acute than that represented by No. 440 was observed in three cases. No. 429, the first of the present outbreak to come to autopsy, was born September 7 and died October 12. The second, No. 436, was born September 20 and died

November 4. The third, No. 447, was born October 17 and died November 29. The three calves were thus respectively 35, 45, and 43 days old at the time of death. The three cases present certain differences among themselves.

No. 429.—All lobes are much enlarged over the normal collapsed size. There is an adhesion of most of cephalic and ventral lobes to ribs and to pericardium. The attachments are easily broken except the adhesion of right cephalic lobe to pericardium which is not separable. Consolidation of all but one-half of right caudal and two-thirds of left caudal lobe. There is a general faint putrefactive odor emanating from the lungs. The affected lung tissue is dark red and sprinkled over with numerous yellow, cheesy foci 2 to 5 mm. in diameter and projecting slightly above the pleura. These foci permeate the lung tissue with the exception of the caudal and azygous lobes in which such foci are few and small. Besides the small foci the right cephalic lobe contains a sequestrum 2 by 2 by 5 cm., yellowish, and firm like rubber. The trachea is uniformly reddened and covered with whitish, viscid flakes.

No. 436.—Extent of lung involvement and size of affected lobes are as in No. 429. The lung tissue resembles that of No. 440. It is sprinkled over densely with lighter grayish foci about 0.5 mm. in diameter. On pressure very little fluid and only a few consistent molds can be expressed from the air tubes. The multiple necroses found in No. 429 are absent. The mucosa of the trachea is uniformly congested, the bronchi deeply so, bordering on hemorrhage. The mucosa has on it some very thin patches of a pseudomembrane.

No. 447.—The involvement is somewhat less extensive than in Nos. 429 and 436. The pneumonic lobes are firm, only moderately larger than the normal collapsed state, uniformly dark red, and interspersed with a few inflated lobules. There is no distinct mottling with lighter dots as in No. 436, but the surface is rather variegated with larger patches of lighter color. Necrotic foci are not found. The trachea is clean and normal to the bifurcation where some flour-paste-like masses of mucopus are lodged. The main bronchi of affected lobes are filled with a similar thick pasty mass but those of the caudal lobes are free.

The microscopic picture in these three cases as constructed from sections of the different lobes is very much like that of No. 440 already described. The consolidation is due to focal infiltration of the parenchyma with the type of cell described under No. 440. Polynuclears are scarce or absent. The minute air tubes contain, besides cell masses, the homogeneous, bluish stained material, and it is in and among this material that masses of minute bacteria occur. The necroses of No. 429 occupy variable areas of lung tissue in which the alveoli have become impacted with the cell type mentioned. In these cases the gathering of plasma cells in the mucous membrane of the air tubes had not yet begun.

Cultures made from different parts of the lungs of the three cases indicated the presence of various species. Present in all three was *B. pyogenes*, especially abun-

dant in No. 429, less so in No. 436, and least so in No. 447. The sections containing the necrotic foci of No. 429 stained according to Gram-Weigert showed large, deep blue spots resolved as minute bacilli and evidently colonies of *B. pyogenes*. Several other types of colonies were found in the cultures. *B. actinoides* could not be detected in cultures from Nos. 429 and 436. Several cultures from No. 447 contained large numbers of colonies of *B. actinoides* but pure cultures failed because of the presence of *B. pyogenes*. Inasmuch as the type of disease was the same in the three cases, it is probable that *B. pyogenes*, being an acid producer, interfered with the development of *B. actinoides*.

Another group of cases is represented by calves in which the disease processes have gone on more slowly and to a much farther stage. The involved tissues have become necrotic and encapsuled, causing firm adhesions of the pleura to the chest wall and pericardium. There is a broad zone of plasma-like cells around the smaller air tubes, varying in size with the age of the calf.

Case 455.—Female calf born July 26; 4 months old lacking 3 days when killed. The attendant reported that this calf was sick with scours in August. Late in September it had a cold and in November symptoms of pneumonia were present. When killed it was very thin, the respirations short and labored. The cough was frequent, the temperature 40°C.

In this case also the pathological changes were limited to the respiratory organs. When the sternum was removed, the lungs collapsed vigorously. The pneumonic process had the usual extent and symmetrical distribution. The appearance of the lobes was much the same. The tissue is a reddish yellow and beset with closely crowded grayish areas, about 2 mm. in diameter, although varying more or less in size. Similar areas are seen on the cut surface. In the right cephalic lobe the grayish areas are larger and tend to coalesce. In the free tip of this lobe there is a group of larger foci about pea size, made up of thin walled sacs containing what appears to be in part necrotic lung tissue, in part mucus and pus. The pleura covering these foci gives rise to delicate fibers attaching lobe to pericardium. The mucosa of the trachea is normal and has lying in it white, curdy, viscid masses 4 to 5 mm. in diameter made up of polynuclear cells, alveolar epithelium, mucus, and some minute bacilli. All the small air tubes in the consolidated lobes when compressed exude thick, glairy, pearly white masses. Microscopically this material is like that in the lower trachea. All thoracic lymph nodes are distinctly larger than normal; on section quite moist and exuding a milky fluid.

The histological picture differs in degree from that of the more acute types. The most striking character is the presence of a zone of plasma cells under the epithelium of the air tubes, varying in thickness and especially broad in some lobes. The smallest air tubes are distorted, or else nearly obliterated so that their presence is only indicated by the plasma cell groups and zones. These cell zones plus

a broadening of the submucous layer probably represent the grayish areas seen from the surface and on section. The lung tissue around and between these zones is either slightly inflated and contains some desquamated cells, or else is collapsed or filled with polynuclear leucocytes. In sections bacteria are not seen, except in one large mass growing into and nearly obliterating the already dilated and deformed tube. In this mass colonies of fine, rather feebly stained bacilli are present. The flora of the pneumonic lobes of this case resembles somewhat that of No. 432. Of the large number of cultures, about thirty in all prepared with minute and pea size bits of lung tissue, all showed development. The colonies in each tube were few in number. *B. pyogenes* predominated in being present in most tubes. A second form, consisting of 2 to 3 mm., smooth, grayish, translucent colonies which tended to flow down the slant, was probably a variety of *B. bovisepticus*. Several other forms were present. *B. actinoides* was detected in only two cultures among other forms. Among several cultures from bits of tissue from thoracic lymph nodes, one contained *B. pyogenes*.

Case 462 (Text-Figs. 1 and 2 and Fig. 12).—Female, born Nov. 4; killed Jan. 7, weighing at this time 102 pounds. It began life with scours which persisted for some weeks. Evidences of respiratory trouble began to appear Dec. 27. Although only a few days over 2 months old when killed, it presented advanced destructive lesions in larger number than any other case of this small outbreak. The pathological changes, if we except the general disappearance of fat deposits, were restricted to the lungs. Both cephalic and ventral lobes and a small adjacent zone of both caudal lobes were involved. The smaller lobes were adherent to the chest wall and pericardium through thin bands severed with some difficulty. In all but the caudal lobes are isolated or agglomerated, whitish, projecting nodes 5 to 10 mm. in diameter. The lung tissue between them is dark red and beset with paler grayish areas 2 to 3 mm. in diameter. The nodes are sacs containing a glistening, pearly white, viscid, flour-paste-like mass, rather sticky and not easily spread on cover-slips. It consists of cellular elements among which are dense masses of minute bacilli. Within this mucoid, whitish coating is a nucleus consisting of a lobulated mass not so white as the mucoid covering, more brittle, and finely spongy. The gross and microscopic characters indicate necrotic lung tissue. The walls of the cavities containing the sequestra are 1 to 1.5 mm. thick, and smooth. Where a number of cavities are close together they evidently communicate, since pressure continues to force the necrotic and mucoid masses out of all the cavities through one opening. Stained films of tracheal mucopus and pus from the necrotic foci show besides cellular elements many minute bacilli frequently in dense masses. The air tubes of the affected lobes contain the same viscid, mucoid, purulent masses found in preceding cases.

The histological changes found in the various lobes do not differ materially from those of the advanced, chronic type. In general there is much collapse of the parenchyma with marked broadening of the alveolar walls due to infiltration of endothelial cell types. The collapsed tissue is focally occupied by dense infiltrations of polynuclear cells into the alveoli. In some lobes the normally distended

alveoli contain sparse collections of desquamated cells and polynuclears. The air tubes in some lobules have a dense enveloping zone of plasma cells. In others it is very slight. Sections through the necrotic foci show sequestra of lung tissue enclosed in a dense zone of plasma cells and more or less connective tissue stroma. Bacteria colonies are not seen.
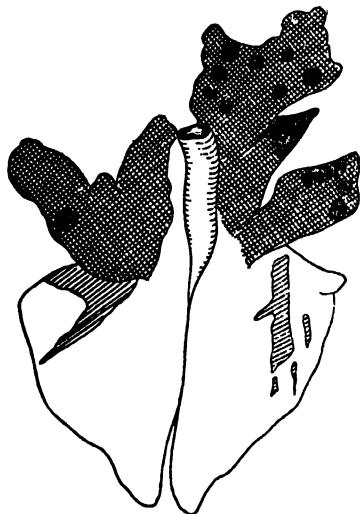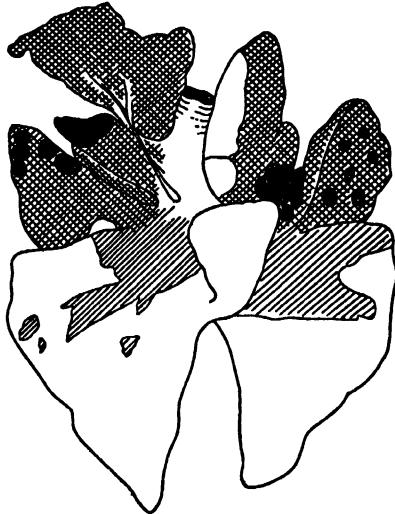
TEXT-FIG. 1. TEXT-FIG. 2.

Diagrams of involved lung tissue, Calf 462.

TEXT-FIG. 1. Dorsal aspect.
TEXT-FIG. 2. Ventral or diaphragmatic aspect.

In the text the lobes are designated cephalic, ventral, and caudal. In the right lung the cephalic lobe is relatively very large and has a separate bronchus. In Text-fig. 2 the small median or azygous lobe is shown. The normal lung tissue is unshaded; the heavily shaded circles represent necrotic tissue; the areas crosshatched designate an older stage than the areas having parallel lines only.

Cultures were prepared from the different lobes on several media as described under Case 440. The extensive necrosis of lung tissue naturally led to the inference that a variety of bacteria would be found. This, however, was not the case. In none of nine horse serum tubes did any liquefaction suggesting *B. pyogenes* occur. In all but one, which remained sterile, *B. actinoides* multiplied in pure culture. Similarly the plain agar tubes containing bits of tissue and blood agar tubes developed pure cultures of *B. actinoides* with two exceptions in which a mold and a fleshy colony appeared.

The following case was the last of this particular outbreak.

No. 508.—Female calf, born May 7. Attendant gives a history of digestive disturbance (scours) beginning a few days after birth and lasting 3 weeks. When first seen June 3, the calf was emaciated, weighing 75 pounds. The respirations were rapid and shallow. Both nostrils were soiled with whitish, mucoid masses. The right ear was held low and was discharging a viscid, pus-like matter. The temperature fluctuated between 39° and 40°C. until June 7, when it was killed.
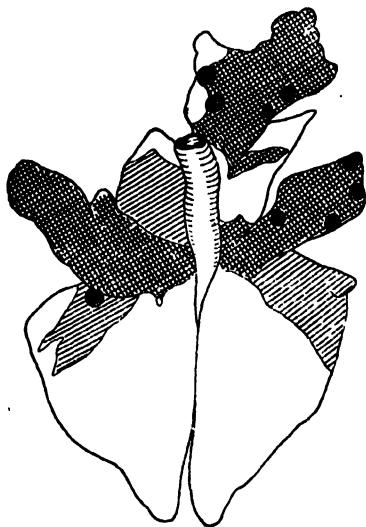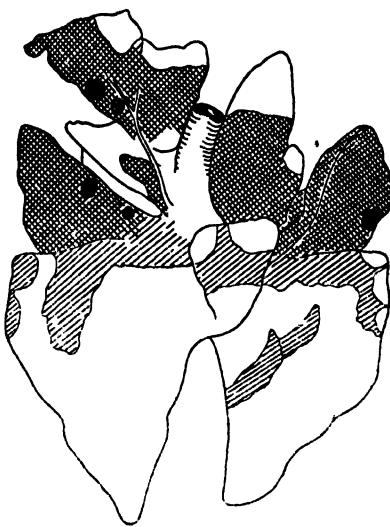
TEXT-FIG. 3.   TEXT-FIG. 4.

TEXT-FIGS. 3 and 4. Dorsal and ventral aspects of lungs of Calf 508. For explanation of the shading see Text-figs. 1 and 2.

The autopsy showed the usual lung involvement (Text-figs. 3 and 4). Three types of lesions are present: (1) the focal necroses; (2) a firm hepatization with slight enlargement of the cephalic and ventral lobes, which are furthermore permeated with grayish foci 1 to 2 mm. in diameter; and (3) a more recent stage, characterized by a uniformly dark red airless condition. The affected lobes are furthermore variegated by air-containing territories. The lesion referable to the right ear was not traced, owing to the injury inflicted by the blow necessary to stun the animal. A small cavity was found in the temporal bone filled with a puriform liquid which may have been associated with the lesion. Films from this fluid contained numerous polynuclears and several kinds of bacteria (diplococci, bipolar and minute bacilli). Cultures from various regions of the diseased lungs were made. In all a variety of bacteria developed, among them *B. pyogenes. B.*

actinoides was detected in all serum cultures. In two tubes the colonies of this bacillus were present in large numbers and among them scattering liquefying colonies of *B. pyogenes*. Pure cultures of *B. actinoides* could not, however, be obtained from these tubes. The sections of material fixed in Zenker's fluid did not show any details differing from those already described.

As an illustration of the condition of the lungs of an animal surviving this type of pneumonia, the following case from the first outbreak is of interest.

No. 6.—This calf was nearly 7 months old when killed. It was born in June, 1916. When received the calf was normal as to temperature, respiration, and pulse. It was thin and hide-bound, but not unusually weak. No early history was obtained. It was killed because unthrifty. The disease was restricted to the right cephalic lobe of the lung. It was adherent to surrounding structures by easily broken fibers. One-half of the lobe is permeated with yellowish white sacs containing a thick creamy fluid. The other (caudal) half of the same lobe presents a grayish mottling of regular pattern. The air tubes of the entire lobe are surrounded by broad, whitish bands, or zones, and contain viscid, glairy molds. The trachea contains a large amount of a viscid, flour-paste-like whitish matter. In sections of the affected lobe the air tubes stand out prominently as thickened tubes. The thickening is due to a broad envelope of plasma cells and new connective tissue around them, while what is left of the parenchyma is slightly emphysematous and contains scattering desquamated alveolar cells (Fig. 13). A number of partly occluding ingrowths into small air tubes are made up largely of an endothelial cell type with an admixture of polynuclears. In a mediastinal lymph node, associated with the diseased lobe, the normal lymphoid cells are almost entirely replaced by the plasma cell type. Cultures from the various lobes developed a rich growth of a bacterium which agreed in cultural characters with the bipolar type of organisms (*B. bovisepticus*). Its virulence towards rabbits was very low. At this time *B. actinoides* was not yet known to the writer. The lesions were ascribed to *B. bovisepticus*.

After an interval of 6 months following the second (1919) outbreak a sporadic case of pneumonia appeared, which is of importance in several directions.

No. 544.—Female calf, born Oct. 6, 1920. It was reported sick by attendant Nov. 18 and transferred to the Institute next day. The animal was emaciated and very weak, unable to stand. The respirations were rapid and shallow. The buttocks were soiled with feces. Temperature on Nov. 19 was between 41° and 41.8°C. during the day. The animal refused all food. Early Nov. 20 the attendant found the calf panting and grunting. It died soon after and was autopsied at 10 a.m.

Besides the extensive pneumonic changes, the only noteworthy lesion is a septic condition of the stumps of both iliac arteries. The lumina are patent and the intima is coated with a thin, grayish, pultaceous layer not removable by washing. One artery contains a grayish yellow, cylindrical thrombus about 2 cm. long. The lungs are extensively hepatized. Even the left caudal lobe is nearly one-half solidified. The cephalic and ventral lobes of both sides have dimensions about twice those of the normal collapsed lung. The hepatization is smooth, grayish red, excepting where the cut bronchi exude whitish, pasty masses. The hepatization of the azygous and the caudal lobes is of a dark red color, less firm. The pleura is free, adhesions absent. Necroses are not detected in any lobe. Both main bronchi contain flakes of thick, glairy matter filling the mouths of many branches. The trachea contains many roundish masses of the same exudate, pea size and coated with froth. Tubes inoculated from various affected lobes; about twenty in all, develop, with one exception, into pure cultures of *B. actinoides*.

The nature of the pathological process was not made clear in the sections examined. The invasion of the epithelium of the ultimate bronchioles shown so clearly in No. 440 could not be seen. The air tubes were plugged with cell masses extending into them from the alveolar ducts. The cells consisted largely of polynuclears, feebly tinted necrotic cells, and cells resembling polynuclears in staining but with roundish nuclei. Throughout the affected lung tissue cells of endothelial and plasma type were abundant and intermingled with the others. Owing to the congested condition of the capillary network their relation to the alveolar walls was not clear. Cloud-like masses of minute bacilli were present among the cell masses and resolved with difficulty into their elements.

To bring this case into relation with those already described it may be assumed that the infecting agent was introduced and disseminated through the lungs in such large numbers and deposited in so many places that the process did not have time to reach the stage of focal necrosis before the calf died.

To the information gained by the two groups of cases in the same large herd may be added some data obtained from the autopsies of calves used for producing small-pox vaccine in the vaccine laboratory of the Massachusetts State Board of Health in Boston. During the year 1908 a small number of pneumonic lungs were found in calves killed after the vaccine had been removed. They weighed between 150 and 200 pounds.

No. A.—Both cephalic lobes are pneumonic. The affected tissue is flesh-red with lighter mottling or else simply collapsed. The air tubes contain molds of creamy pus. Sections show the stage of general polynuclear infiltration of paren-chyma and the filling up of minute air tubes with the same kind of cell masses.

In one section there is a cellular irruption into an air tube and in the proliferated mass are colonies of minute bacilli.

No. B.—Both cephalic and ventral lobes are consolidated, and there is also a large focus in the left caudal lobe. Involved tissue is grayish red to grayish yellow, delicately and regularly mottled. The air tubes are distended with whitish, viscid plugs. The process in the focus in the caudal lobe is freshest, that in the free tips of the ventral lobes oldest. In general the histological picture is that of collapse, and infiltration with polynuclears. In one section there are two contiguous foci of cell exudation and hemorrhage undergoing necrosis. Many colonies of fine bacilli are in the periphery of these foci (Fig. 10). These cases, as far as the information goes, suggest an underlying process like that initiated by *B. actinoides*.

The Pathogenic Action of Bacillus actinoides.

The bacteriological study of spontaneous cases pointed to *Bacillus actinoides* as the primary inciting agent of the bronchopneumonia with *Bacillus pyogenes* and more rarely, in older calves, *Bacillus bovisepticus* settling down in the occluded air tubes and necrotic lung tissue. The next step was to determine whether cultures of this bacillus, under suitable conditions, could produce lesions like those found in spontaneous cases. In the following pages is given a brief statement of observations made on inoculated calves, since small mammals had thus far shown themselves quite insusceptible. Some of the calves were inoculated subcutaneously, some intravenously, others into the trachea. The area of the operation was shaved, cleansed with water and alcohol, and painted with tincture of iodine. In the intratracheal injections, a small incision was made through the skin over the trachea before inserting the needle.

No. 467.—Bull calf, born Jan. 21, 1920. Dam had placenta retained at birth and swab from uterus as well as agglutination test was positive for *B. abortus*. Temperature, taken twice daily, fluctuated between 38.5° and 39°C. until Feb. 3, the day of inoculation.

The turbid condensation water of a horse serum culture of *B. actinoides* from Calf 462, 5 days old and under artificial cultivation since Jan. 7, was drawn off, making about 1 cc. in all. This was increased to 3 cc. by the addition of sterile bouillon. The fluid was injected into the subcutis in front of right shoulder. The temperature rose to 39.8°C. within 3 hours and remained at the same level next day. On this day a local swelling appeared over the point of inoculation about 1½ inches in diameter and ¼ to ½ inch thick. The temperature fell below 39°C. on Feb. 8. The swelling increased slightly.

On Feb. 19, a second inoculation was made, this time into a jugular vein with the same strain used for the first, subcutaneous inoculation. The condensation water was ground slightly in a crucible to break up the flocculi and calf serum water was added to dilute the suspension. The temperature rose to 40°C. within 3 hours and gradually fell to normal during the night. No further elevation occurred. The calf was killed Mar. 4. The weight had risen from 88 to 125 pounds. The local swelling was about the size of a small hen's egg. The calf was stunned with a heavy blow, a clamp quickly placed on the trachea, and the neck vessels were severed. During this operation the local swelling ruptured and several cubic centimeters of a thick, whitish mass, like flour-paste, were discharged. The autopsy showed normal organs, except for two very small foci of collapse, one in the right cephalic, the other in a caudal lobe of the lungs. The local swelling is adherent to the overlying skin, but readily dissected from the subjacent fascia. The under surface of the capsule is sprinkled with minute hemorrhages. It is 3 to 4 mm. thick, pearly white on section and enclosing a cavity partly filled with a soft, odorless mass like flour-paste. The inner surface of the capsule is dark red to hemorrhagic and delicately mammillated. The cavity has small recesses or pockets. The puriform contents are readily removed from the entire wall. The associated prescapular lymph node is about one-fourth larger than the opposite node and on section smooth and quite juicy. The contents of the abscess consist of cells, only a few of which retain the stain. These are mononuclear. Scrapings from the capsule show a mixture of polynuclear and mononuclear elements and occasional cloud-like masses of minute bacilli. Some of the mononuclear cells contain groups of minute bacilli. *B. actinoides* was recovered from the abscess both on horse serum and on agar plus blood. In one of the latter the growth appeared richer than usual and was found by inoculation into two guinea pigs and by microscopic examination to contain *B. abortus*. The presence of *B. abortus* is accounted for by the infection of the dam which had been demonstrated by suitable tests after the birth of the calf.

No. 336b.—Bull calf, born Feb. 28, 1920. On Mar. 9, it weighed 111½ pounds. A horse serum culture from Calf 462, 5 days old, was used. The condensation water, which contained a dense suspension of flocculi visible to the naked eye and shown to be pure by the microscope, was removed to a sterile tube, 5 cc. of bouillon were added, and the whole was thoroughly shaken. The flocculi were still visible after the shaking. The suspension was injected into a jugular vein at 4 p.m. The temperature rose from 38.8° to 40.6°C. in 5 hours and gradually dropped to 38.9°C. during the night. The calf was kept under observation for 36 days. During this period the temperature remained between 38° and 39°C. and the calf's general condition was normal. On the 36th day it weighed 145 pounds and was sold for slaughter.

No. 479b.—Bull calf, born Feb. 26. Inoculated on Mar. 9, when 12 days old. A first culture from the local lesion of Calf 467 on agar plus blood, 5 days old, was used. The surface growth was transferred with a platinum loop to 5 cc. of bouillon until a fine suspension was produced. This was made up of excessively fine

granules when viewed in a strong light. It was injected into a jugular vein. Calf weighed at this time 97½ pounds. The temperature rose from 38.7° to 40°C. within 5 hours. It was only a few tenths above normal next day. The calf continued normal in condition. The temperature remained normal.

On Mar. 24, 15 days after the intravenous injection, a second inoculation was made, this time into the trachea. The condensation water of three horse serum cultures of *B. actinoides*, 4 days old, from Calf 467 was removed to a sterile tube and enough bouillon added to make 12 cc. in all. 5 cc. of this were injected. Within 24 hours a swelling appeared over the trachea, of undefined borders, probably 6 inches long and 2 inches broad. After several days this swelling was somewhat reduced and was now found to be made up of two indurations, one over the point of injection, the other lower down at root of neck. Apr. 5. The right leg was shown to have restricted motion and to be painful when handled. Apr. 15. The right shoulder appeared a trifle larger than the left and tender. The lameness increased. The calf, showing no general symptoms, was killed Apr. 20, 27 days after the intratracheal injection. The only lesions found were the local swellings. One about 2 inches in diameter is situated just caudad of the larynx; a second, 4 inches in diameter, just caudad of the first. Both are firm, tense, slightly fluctuating. They are covered by muscles of the neck and attached to the cervical portion of the thymus. Both are sacs with walls 5 to 10 mm. thick, smooth interiorly, and filled with a soft odorless mass, resembling cottage cheese. They communicate with each other through a narrow opening. The contents consist of lymphocytes and polynuclears, the former greatly predominating. Both kinds of cells largely refuse the stain. There are also filamentous masses, probably necrotic fibers. In the larger abscess a strip of necrotic tissue was found in the caseous contents about 4 inches long, representing the remnants of a vessel about 2 mm. internal diameter. After washing away the pus the external surface was found covered with small papilla-like projections.

On the prominence of the right shoulder joint there is a swelling about the size of an egg, easily dented by pressure. The tumor is situated between the tendinous ends of the muscles inserted in the scapula. There is marked thickening of the intermuscular fascia radiating from the capsule of the tumor to a depth of several inches. On incision of the thick capsule, an irregular cavity is exposed, with contents mucoid, whitish, quite unlike those of the tumors over the trachea. However, films show the same cell elements, the difference being due to the advanced necrosis of the contents of the other abscesses.

Sections of the abscess walls show a mass of muscular and connective tissue with marked hyperplasia of the connective tissue and some fibrin. There is also a general infiltration with cells of endothelial character. The pus attached to the inner wall consists wholly of cells of endothelial type. Some of these are filled with minute bacilli. There are also clouds of free bacilli feebly stained and easily overlooked. Cultures prepared from both abscesses over the trachea develop into pure growths of *B. actinoides*. No cultures were made from the abscess on the shoulder. The respiratory tract as well as the remaining viscera was normal.

No. 495b.—Female calf, born Apr. 15, 1920. On May 11, 10.30 a.m., when 26 days old and 88 pounds in weight, it was inoculated into the trachea. The condensation water of six horse serum cultures from Calf 479b, 5 days old was drawn into a fresh tube and 6 cc. of bouillon were added, making 13 cc. of heavily, clouded fluid. 8 cc. of this were injected. Animal coughed occasionally after
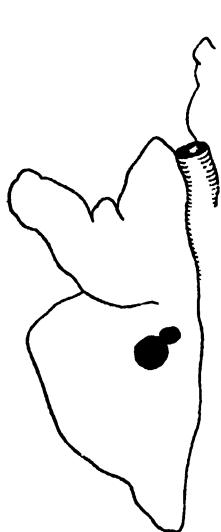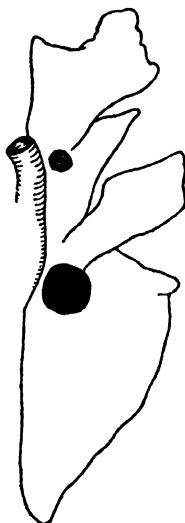
TEXT-FIG. 5.   TEXT-FIG. 6.   TEXT-FIG. 7.

Effect of the intratracheal injection of cultures of *B. actinoides*.

TEXT-FIG. 5. Necrotic focus in left caudal lobe of Calf 520.
TEXT-FIG. 6. Similar focus in right cephalic lobe of Calf 474b.
TEXT-FIG. 7. Two necrotic foci, one in cephalic, the other near root of right ventral lobe of Calf 495b.

the operation. The temperature rose about 1°C. during the day and was down again the next day. 3 days later the temperature reached 40°C. and fluctuated between 40.2° and 40.7°C. for 6 days, then dropped to 38.3° to 38.7°C. The calf weighed 99 pounds on May 27, having gained 11 pounds in 16 days. It was killed on this day.

The digestive tract is normal throughout. In the respiratory tract there is a bit of semitranslucent mucus on one vocal cord. In the lower trachea there is a similar mass, pea size, made up of alveolar epithelium and polynuclear leucocytes. There are two groups of focal necroses in the lungs (Text-fig. 7). On the dorsal ridge of the right caudal lobe, near root of the right ventral, there

is a small, whitish, opaque, subpleural mass.   On palpation, other firm nodules are felt embedded, about half a dozen in all.   On section the contents show as thick, cheesy cores in dense capsules.   The tissue between them is still air-containing.   There is no fresh pneumonia or collapse around them.   A similar cheesy nodule is in the right cephalic lobe, embedded in emphysematous tissue. Besides these two necrotic foci there are several collapsed lobules in different lobes. Lymph nodes of thorax are normal.   There are 10 cc. of clear fluid in pericardial sac.   Both kidneys are spotted everywhere with whitish areas, discrete and con-fluent, occupying fully one-half of the total cortex.   These spots are the bases of cones of fan-shaped outline on section, extending to the medulla.   The substance of these foci is glistening, smooth, almost like cartilage in appearance.   Urine taken from the bladder soon after death is very pale, clear, faintly alkaline, spe-cific gravity 1,004.   Boiling after adding 1 to 2 drops of acetic acid yields a very faint cloud.   The liver is slightly fatty.

Sections of the necrotic foci in the lungs show centrally a nucleus of dead lung tissue in which the alveolar structure is still preserved (Figs. 15 and 16).   These nuclei are 1.5 to 2 mm. in diameter.   In the central core the alveoli are nearly empty.   In the peripheral zone, they are filled with a fine granular material in which many cells are embedded.   Immediately surrounding the necrotic center is a broad zone in which the lung structure is occasionally seen, but is chiefly replaced by cellular debris embedded in a granular matrix.   Dense groups of minute bacilli are in the outer layers of this zone where many alveolar cells are still recognizable.   Outside this zone is a layer of fibroblasts and newly formed capillaries.   The whole is enclosed in a mantle of lymphoid cells, occupying the meshes of the compressed lung tissue.   In the vicinity of the necrotic focus there are zones of lymphocytes around the ultimate and next larger bronchioles, associ-ated with collapse and broadening of alveolar walls through cell infiltration.   In some air tubes small groups of polynuclears are seen.   Sections of the kidney lesions show interstitial hyperplasia with shrinkage and disappearance of glomer-ular tufts, dilatation of some convoluted tubules and disappearance of others. Cultures made from the necrotic lung foci both on horse serum and blood agar developed the characteristic appearances of *B. actinoides*.   Only one contained also a fine filamentous growth, probably a streptothrix.   All of sixteen cultures prepared from kidney tissue remained free from growth.

No. 474b.—Black and white bull calf, born Apr. 21, 1920.   June 3.   The calf weighed 135 pounds.   The condensation water of three horse serum cultures, 4 days old, showing a dense crop of flocculi was withdrawn into a sterile tube, about 4 cc. in all.   This was injected into the trachea, and without removing the needle, about 6 cc. of sterile salt solution were injected through it.   After the calf had been placed on its feet it coughed some, ejecting a fine spray.   Following the injection the temperature rose about 1.5°C.   It was normal next day.   The calf was killed June 21, 18 days after the inoculation.   The autopsy showed normal conditions with the following exceptions.   In the abdominal cavity there are

about 50 cc. of a clear, slightly yellowish fluid which coagulates into a jelly-like mass on standing. Between the vocal cords, along the trachea, and at the root of the right supernumerary bronchus are small masses of a semi-opaque, mucoid substance, consisting of polynuclears, alveolar, and endothelial elements embedded in mucus. The lungs show quite generally over all lobes scattering, very small, dark reddish collapsed or pneumonic foci from mere points to 1 mm. in diameter and of various shapes. In the right cephalic lobe near root is a consolidation about 3 cm. in diameter, but more or less squarish and extending through depth of lung tissue (Text-fig. 6, page 457). It feels lumpy. On section the lumps appear as spherical, firm, grayish foci, about 1 cm. in diameter and centrally necrotic. There is congested lung tissue between them. Each focus is made up of a very firm, almost cartilaginous capsule. Within is a viscid, very thick, whitish mass, which in the larger foci contains in it a more grayish, spongy nucleus found to be necrotic lung tissue. The layer around this contains many mononuclear elements whose cytoplasm, in many cells at least, is filled with fine rods. Masses of similar rods are free in the stained film. The necrotic masses are odorless. Cultures made from the necrotic foci on horse serum showed the characteristic flocculi in 3 days. All contained in addition molds with delicate mycelium. Pure subcultures of *B. actinoides* were obtained, however, from surface colonies. Cultures on blood agar were also prepared. Some were pure growths of *B. actinoides*, others contained one or several other types of colonies. At the same time, cultures were prepared from the air-containing, normal lung tissue by introducing into culture tubes small bits of lung tissue. Molds, liquefying colonies, and several other types of colonies appeared in the tubes.

No. 504b.—Black and white female calf, born June 10, 1920. Weight, June 21, 110 pounds. On June 28, the condensation water of six serum tubes of *B. actinoides* from Calf 474b, 3 days old, was brought together in a sterile tube. The heavily clouded fluid, 5 cc. in all, containing flocculi was injected into the trachea at 3 p.m. The temperature rose about 1.5°C. within 4 hours, then slowly subsided. No abnormal conditions appeared, and it was killed 23 days after inoculation. At this time it weighed 136½ pounds. No lesions were found in the respiratory tract or elsewhere.

No. 520.—Guernsey calf, male. Born Oct. 8, 1920. Weight on Oct. 22, 83½ pounds. On Oct. 27, four horse serum tubes inoculated from cultures of Calf 474b, 4 days ago, were used for intratracheal injection. The condensation water was drawn up into a sterile tube, a little salt solution being used to wash it out more thoroughly. 5 cc. of a moderately clouded fluid were collected in this way. This was injected into the trachea at 10.30 a.m. Before removing the needle, 10 cc. of sterile Ringer's solution were injected. The temperature rose from an average of 38.6° to 41°C. 7½ hours after the injection and gradually fell during the night—the temperature being taken every 2 hours—to 38.8°C. next morning. Following the injection the calf began to appear depressed, the respirations were slightly irregular, about 90 at 2.30 p.m. Attendant noticed a slight chill between 4.30 and 5.30 p.m. The temperature did not go above normal again and the

calf, showing no signs of any disturbance thereafter, was killed Nov. 12. The weight on this day was 96 pounds. The organs were normal with the following exceptions.

Beginning about 5 cm. below lower margin of the larynx, the mucosa covering six intercartilaginous spaces of the trachea is dark red over each space. There are no signs of swelling. In the left caudal lobe a firm mass is buried within air-containing, slightly emphysematous lung tissue (Text-fig. 5, page 457). It is about 1 cm. in diameter. Soft, smooth, flour-paste-like material oozes out from a slight incision. The mass is placed in Zenker's fluid. A film of this thick mass shows necrotic cells and no bacteria. There is a second 2 to 3 mm., firm, yellowish white focus near the first. It contains a nucleus of necrotic lung tissue, enveloped in the same material found in the larger focus. Films of this contain large numbers of minute bacilli among cells of endothelial and leucocyte type which hold the stain as if still living. Cultures were not prepared from this case, owing to the scanty material and the need for histological examination of the lesions. This did not, however, show more than has been given from the other cases in which intratracheal injection of *B. actinoides* produced focal necrosis. The central nucleus of the necrotic tissue was surrounded by a layer of polynuclear leucocytes and outside this a connective tissue capsule was forming. Between these two layers, within a narrow zone, colonies of minute bacilli were abundant. Many groups of bacilli were within the cytoplasm of cells.

The results of the inoculation of cultures of *Bacillus actinoides* may be briefly summarized. The subcutaneous injection leads promptly to a large swelling which becomes very firm. After several weeks the mass of the swelling becomes necrotic, the contents caseous, and a thick, firm wall forms with hyperplasia of the connective tissue in the immediate environment of the focus. Softening of the capsule, ulceration of the overlying skin, and discharge outward take place within 4 weeks. In several cases, in which the injections were into the trachea, a small amount of the culture fluid entering, accidentally, the tissues overlying the trachea led to large abscesses. The swelling is primarily due to an increase of mononuclear, endothelial-like elements, with later invasion of polynuclears. The final product is a soft mass, like cottage cheese in appearance, consisting of small lumps, the whole embedded in a thick mass, smooth, glistening, like flour-paste in consistency. In one abscess the remnant of a vessel, 4 inches long, was embedded in the mass. Clouds of minute bacilli are found in the zone outside the necrotic mass where the cellular elements are still stainable. In one case (No. 479b) a second (metastatic) abscess developed at some distance from the site of inoculation.

Injection into the circulation in one case failed to produce any lesions. The second case was sold in excellent condition, probably without lesions. Injection into the trachea failed in two out of five cases to produce any appreciable changes in the lungs. In the remainder there developed small necrotic foci, single or multiple, identical both macroscopically and microscopically with those occurring in spontaneous cases.

The distribution of the induced lesions did not agree entirely with that of spontaneous cases, thus meeting in part the possible objection that the lesions might have been due to naturally acquired infection. The difference in localization was probably due to the fact that the calves were lying on their backs when the culture fluid was injected. This abnormal position may have led to a different drainage of the injected fluid from that occurring in spontaneous cases.

In one instance (No. 495b) the lung lesions were associated with multiple focal scleroses of the kidney cortex.

The culture used was obtained from Calf 462 and passed in succession through Nos. 467, 479b, 495b, 474b, and 520. This was done with the expectation that the virulence would maintain itself. From every one of the above, excepting No. 520, pure cultures were recovered. None were made from the last case.

It will be noted that the intratracheal inoculations produced what was called above the first stage of the disease only. The lesions were restricted in extent and the secondary lesions due to dissemination of the bacilli from the primary necrotic foci did not take place. Perhaps some special depressing conditions may be needed to continue the disease into the clinical stage.

In contrast to the very acute, both destructive and tissue-stimulating action of *Bacillus actinoides* in the subcutis of calves is the following negative action on one sheep.

No. 164.—Barren ewe. On May 11, 1920, the same suspension of cultures from Calf 479b used on No. 495b was injected under the skin in front of left shoulder. Neither local nor thermic reaction followed. The experiment was closed June 1.

Correlation of the Pathological and Bacteriological Data.

The foregoing observations and experiments demonstrate the existence of a specific bronchopneumonia causing mortality in calves chiefly in the 2nd and 3rd months of life. In animals which die within the 1st month other agencies due to fetal conditions and to infections acquired during or soon after birth may involve the lungs. Surviving cases affected with a chronic pathological condition of one or more lobes, characterized by purulent bronchial exudation, abscess formation, and fibrosis may be met in the 4th to the 6th month or even later. As a rule, the disease invades both lungs symmetrically. The parts affected first are the smaller cephalic and ventral lobes, more particularly the dependent portions (Text-figs. 1 to 4). With the progress of the disease the involvement moves upwards towards the dorsal border of these lobes and backwards into the azygous lobe and the caudal lobes. Death takes place when one-half of the latter have become airless. The pleura is involved only where necroses extend to the surface. Here adhesions to surrounding structures form from the capsules of the resulting abscesses.

Several kinds of lesions are presented in the ordinary acute case. The distal two-thirds of the cephalic and ventral lobes are, as a rule, considerably enlarged beyond the normal collapsed state, very firm, dark or light reddish in color. Regularly sprinkled in this ground are grayish, 1 to 2 mm. areas. The proximal third of the same lobes and the affected regions of the caudal lobes are but little enlarged, uniformly dark red, less firm, and without the grayish mottling. Careful search, by manipulation if necessary, reveals scattering firm masses, sometimes deeply embedded, more commonly extending to one or both lung surfaces. They vary from 2 to 10 mm. in diameter. Several may coalesce. They consist of a pearly, dense capsule containing necrotic lung tissue, enveloped in a layer of viscid pus. These sequestra are with rare exceptions located in the cephalic and ventral lobes. They are probably among the oldest lesions. They and the mottled pneumonic regions which are always associated with them are either developed at the same time or else the necroses are the source of the infection which produces the pneumonic condition. The smooth dark red pneumonia is secondary to the other lesions,

probably through the agency of aspirated purulent exudates. The distribution and extent of the three conditions are shown in the text-figures. The pneumonic condition is usually not universal in the affected lobes. Small or large air-containing territories may occur in them, chiefly along the free margins of the lobes. The one universal characteristic lesion is the filling of the air tubes of the affected lobes with a thick, viscid, glairy, white, mucopurulent matter.

The microscopic characters of the different stages are fairly well definable. The formation of the necroses (Figs. 12, 15, and 16) has not been traced, owing to lack of material in the early stages. Associated with or following these, there is a filling up of the alveoli with several cell types to form the mottled pneumonic territories. At the same time the ultimate bronchioles and alveolar ducts may become involved. The entire parenchyma becomes filled with mononuclear elements, probably a mixture of alveolar cells, endothelial and lymphoid cells. The polynuclear leucocyte is absent in this stage. The smooth, fresher pneumonic condition which develops later is due to partial or complete filling up of alveoli and bronchioles with polynuclear leucocytes. In these the epithelium remains intact. With this stage there appears the zone of plasma cells around the air tubes filled with cell debris (Figs. 7 and 13). Numerous polynuclear leucocytes are found moving outward through the epithelium into the lumina. This accumulation of plasma cells is probably due to the cell debris in the lumina of the air tubes. They are present after a certain time whether one or several species of bacteria are at work. That they are the result of the stimulus exerted by toxins and other products absorbed from the disintegrating cellular plugs in the lumina seems to be at present the most plausible explanation of their presence. The minute grayish dots permeating the older pneumonic lesions, which are so striking in the fresh lung of most but not all cases, have not been associated definitely with any microscopic details. They may represent the earlier proliferative lesions in the ultimate bronchioles in some cases and the gathering of plasma cells in later stages.

Before summarizing the results of the bacteriological studies it should be stated what is apt to be forgotten in an interpretation of results, that the normal lungs of calves, and other species as well,

contain a considerable variety of living microorganisms. When bits of normal lung tissue are placed in culture tubes, growth appears quite invariably. Spore-bearing bacilli, various kinds of molds, and streptothrix are among the commonest forms detected. In the isolation of *Bacillus actinoides*, on agar, bits of lung tissue are essential to growth. On coagulated serum tissue is not necessary. It is somewhat surprising that so many cultures obtained from bits of diseased lung tissue were pure cultures of *Bacillus actinoides*. It would seem as if under the influence of the violent tissue reaction the banal forms found in normal lungs are largely destroyed.

The bacteriological examination has shown a variety of results, depending on the stage of the pneumonic process, the rapidity of its development, and whether the animal died or was killed. In the 1919 to 1920 epidemic, twelve cases were investigated. *Bacillus actinoides* was the only cultivable organism present in five of these. It was present but associated with other pathogenic species in four more. It was not detected in the remaining three. *Bacillus pyogenes* was present in seven cases but not in pure culture. *Bacillus bovisepticus* was present in three or four cases, always with a variety of other species. Of the five cases in which *Bacillus actinoides* was exclusively present, two had died and three were killed. Of the four in which it was associated with other species, three were killed and one died. Of the three in which it was missed, two died and one was killed. A study of the brief protocols suggests that *Bacillus actinoides* was missed through overgrowth with other species in certain very acute dead cases and in the more chronic surviving cases.

In the tissues of the diseased lungs *Bacillus actinoides* is found in the peripheral layer of lung sequestra, in colonies in the cell masses occupying alveoli and alveolar ducts (Figs. 8 to 11), and in the proliferated epithelium obstructing the bronchioles less than 0.1 mm. in diameter (Figs. 1 to 7). This latter process which is rather unique and which has not thus far been described in the pathology of pneumonias was especially well brought out in sections of No. 440, but it could be detected in most other cases after more or less searching. In the invaded epithelium *Bacillus actinoides* appeared with the sheath or capsule around it. In other situations it was usually free from capsules and appeared in dense, cloud-like colonies. It should

be stated here that, with rare exceptions, air tubes over 0.1 mm. in diameter had their epithelium intact. The cell masses with which many were filled—the glairy, mucopurulent contents—consisted of cell debris moving up from the ultimate bronchioles and the parenchyma.

After injection of pure cultures into the trachea *Bacillus actinoides* has thus far produced only the early stage of necrosis (Text-figs. 5 to 7 and Figs. 15 and 16). The diffuse secondary and later lesions characterizing the spontaneous fatal and very sick cases killed were absent. The relation of *Bacillus actinoides* to the primary necrotic lesions is thus placed beyond doubt. That it is also responsible for the general pneumonic involvement is not proved by experiment but made highly probable. Neither *Bacillus pyogenes* nor *Bacillus bovisepticus* is responsible, since these easily cultivated bacteria were absent in five advanced cases. Considerations based on bacteriological and histological studies are sufficient to throw them out of the other cases in which they were found, except in the rôle of continuing a disease fully under way by multiplying in the necrotic tissues and the bronchial cell debris. If *Bacillus pyogenes* and *Bacillus bovisepticus* are not responsible for the diffuse pneumonic lesions, then either *Bacillus actinoides* or else some non-cultivable, unrecognized microorganism is. The writer has throughout assumed that *Bacillus actinoides* is the sole responsible agent.

A study of the mode of response or reaction of the tissues to *Bacillus actinoides* has not come within the scope of the present investigation. The finding of some small susceptible species is necessary to provide material for such study. In general the reaction in the lungs is at first associated with mononuclear cell types, either endothelial or lymphoid or both. In later stages polynuclear cells completely dominate the process. This change may be due to an immunity reaction. Many things point to this explanation. Thus the later smooth pneumonic condition is always associated with the polynuclear cell type. In those animals in which the process halts with the necroses, the remaining parenchyma contains only polynuclears in focal distribution. Moreover, *Bacillus actinoides* is only very rarely detected with the microscope in the lesions associated with polynuclear cells, although cultures reveal its presence. This rarity suggests that the active stage of multiplication is over.

The various factors entering into the process such as quantity and dissemination of the original infecting material, relative susceptibility of the host, and the rapidity with which immune forces are called forth determine the extent and rapidity of the early, usually necrotic changes and the promptness of dissemination of the virus from such necrotic foci over new territories. Added to these factors are species of bacteria, chiefly *Bacillus pyogenes* and *Bacillus bovisepticus*, which may graft themselves on the diseased tissues. In thus restricting *Bacillus bovisepticus* to a secondary position, the author does not imply that there may not be virulent races of *Bacillus bovisepticus* capable of initiating outbreaks of pneumonia.

Aside from Case 6, no detailed description of the cases occurring in the 1917 outbreak, which was the basis of an earlier publication,[1] has been given. This group of cases had not been worked up with so much care and in such detail partly because the very first case which came to autopsy yielded pure cultures of *Bacillus bovisepticus* and the developing epizootic was considered due to this bacillus. With the succession of cases, the appearance of a polymorphic bacillus and its association with a closely resembling species, *Bacillus pyogenes*, rendered orientation very difficult until a pure culture of *Bacillus actinoides* was made to grow indefinitely in subcultures and its forms clearly differentiated from *Bacillus pyogenes*. A repeated study of the accumulated data has shown such a parallelism between the 1917 and the 1919 cases in gross and minute anatomy and histology of the affected lungs and in the bacteriology that any review of these early cases would be in the main a repetition of what precedes. The underlying etiological factor and the processes it initiates are the same in both groups of cases.

A study of the epidemiology of the pneumonia due to *Bacillus actinoides* needs much additional material. The source of this microorganism is not yet defined. It will, however, be safe to regard partly recovered older animals of the same species as the purveyors of the virus. The termination of certain lesions in necrosis followed by copious discharge from the resulting abscesses opens the way for the virus outward. It is probable that unrecognized cases occur in which the process does not go beyond localized necrosis of small territories. Such cases may serve in maintaining the mild disease

until the cold season favors the secondary extension of the lesions and brings acute clinical cases to the surface. This view is supported by the production of necroses experimentally in calves without any appreciable rise in temperature or other suspicious deviations from health.

Owing to the meager descriptions given in reports of outbreaks of pneumonia in calves, it has been impossible to identify this form of bronchopneumonia in earlier writings. An attempt was made in the article on *Bacillus actinoides*[1] to correlate the latter with Lignières' actino-bacillus which produces subcutaneous and other abscesses but not pneumonia. A continued study of *Bacillus actinoides*, however, does not make the relationship seem any more real. It remains to study calf pneumonias in both anatomical and microbiological directions afresh, since the existence of several etiological types of lung disease is probable. The anatomical and histological study of these pneumonias is important, since information gained in this way tends to restrain hasty conclusions concerning the etiology where several different species of bacteria may find opportunity for multiplying in the lungs in the course of the disease.

CONCLUSIONS.

A bronchopneumonia of calves in the early months of life is described and its etiology associated definitely with a minute bacillus, *Bacillus actinoides*. *Bacillus pyogenes*, *Bacillus bovisepticus*, and, less frequently, staphylococci and streptococci may appear later in the affected lungs.

Subcutaneous injections of cultures of *Bacillus actinoides* produce large indurations ending in necrosis. Similarly intratracheal injections produce circumscribed necroses of lung tissue. The cultivation of *Bacillus actinoides* and its morphological peculiarities have been sufficiently described and illustrated in an earlier publication[1] to ensure success on the part of those who attempt to isolate it.

The writer is indebted to Dr. R. B. Little for assistance in bringing together the clinical data.

EXPLANATION OF PLATES.

PLATE 42.

FIG. 1. Section of lung tissue, Calf 440, showing dilatation of a small bronchus and complete filling up of the lumen with the characteristic exudate. The epithelium has disappeared from most of the circumference. The exudate is intimately associated with the subepithelial tissues. The parenchyma around the bronchus is partly collapsed, partly pneumonic. The capillaries are distended with corpuscles, and the alveoli are filled with cells of endothelial and lymphoid type. See Fig. 4 for magnification of a part of this exudate. × 240.

PLATE 43.

Both figures from Calf 440.

FIG. 2. An air tube about 0.1 mm. in diameter with ingrowth of cellular masses, which are filled with bacilli, as shown in Fig. 5. The section passes through a dividing bronchus, of which one branch is involved. Remnants of the epithelial cells are traceable in the irrupted mass which contains cells with round, pycnotic nuclei. × 240.

FIG. 3. Transection of an air tube about 0.3 mm. in diameter. The exudate is intimately connected with the subepithelial tissue of the bronchus in two places. Elsewhere the epithelium is still *in situ*. The exudate is of the same character as in Figs. 1 and 2. It is permeated with cloud-like masses of minute bacilli showing only as slightly darker patches in the photograph. × 310.

PLATE 44.

FIG. 4. A portion of the diseased bronchus of Fig. 1, enlarged × 1,000. The bacillar and capsulated or club-shaped forms are present, filling the cytoplasm of the cell mass which has nearly plugged the lumen of the small bronchus.

FIG. 5. A portion of the diseased bronchus of Fig. 2, enlarged × 1,000. The bacillar, cloud-like masses have filled the cytoplasm of the cells as in the preceding figure. The club-shaped forms are shown in the lower right-hand region in many cross-sections and rather sparsely elsewhere in the bacterial masses.

PLATE 45.

FIG. 6. From Calf 440. Section through a small bronchus, of which the epithelium along one margin (to the right in the figure) is still *in situ*. The other margin is obliterated by an ingrowth which nearly occludes the lumen. This ingrowth is densely permeated with *B. actinoides*. The bacilli appear chiefly as very short rods of irregular size and form throughout the section not taken up by the bronchial epithelium. × 1,000.

FIG. 7. From the same lung. The small air tube is nearly occluded by the mass of cells on the left. This mass is permeated densely with *B. actinoides* in bacillar form. In addition, larger circular areas, suggesting capsulated cocci, are sprinkled through the mass. These are identified as club-shaped forms of *B. actinoides* cut across. × 1,000.

PLATE 46.

FIG. 8. Section through the parenchyma of the lungs, Calf 440. The alveolus is filled with cells. In the center of the cell mass is a colony, or floccule, of *B. actinoides*. × 1,000.

FIG. 9. Two alveoli, each containing a floccule. The clubs are shown imperfectly in optical cross-section. × 1,000.

PLATE 47.

FIG. 10. Section from an old case, designated as Calf B on page 454 of the text showing two foci in which are cloud-like colonies of minute bacilli, appearing as irregular darker patches in the figure. × 1,000.

FIG. 11. Section from the lungs of Calf 440. In the upper left-hand corner is a large cloud-like colony of bacilli. Other colonies in the field are out of focus and appear as slightly darker patches. × 1,000.

PLATE 48.

FIG. 12. Sequestrum from the lung of Calf 462, slightly dislodged. The outlines of the alveoli may still be distinguished in the upper portion of the necrotic mass. The border of the wall or capsule above, which suggests epithelium in the figure, is compressed tissue made up of fibroblasts and plasma cells. × 25.

PLATE 49.

FIG. 13. Section from the lungs of No. 6. The more or less deformed bronch are filled with polynuclear leucocytes and surrounded by a broad zone of plasma cells and fibroblasts. × 925.

FIG. 14. Section of lung tissue from Calf 446. This case is somewhat older than No. 440. Near the center of the figure is a more or less deformed bronchiole containing polynuclear leucocytes. The lung tissue in a broad zone around this tube is occupied by groups and masses of plasma cells and polynuclear leucocytes. × 925.

PLATE 50.

FIG. 15. Represents a necrotic focus from the lungs of Calf 495b (see Text-fig. 7) which received an intratracheal injection of *B. actinoides*. × 60.

FIG. 16. Enlargement of the zone just outside the necrotic mass shown in Fig. 15. Irregular groups of *B. actinoides* occupy this zone. The large group on the right has only a few bacilli in focus, whereas the central group is fairly distinct. × 1,000.

(Smith: *Bacillus actinoides.*)
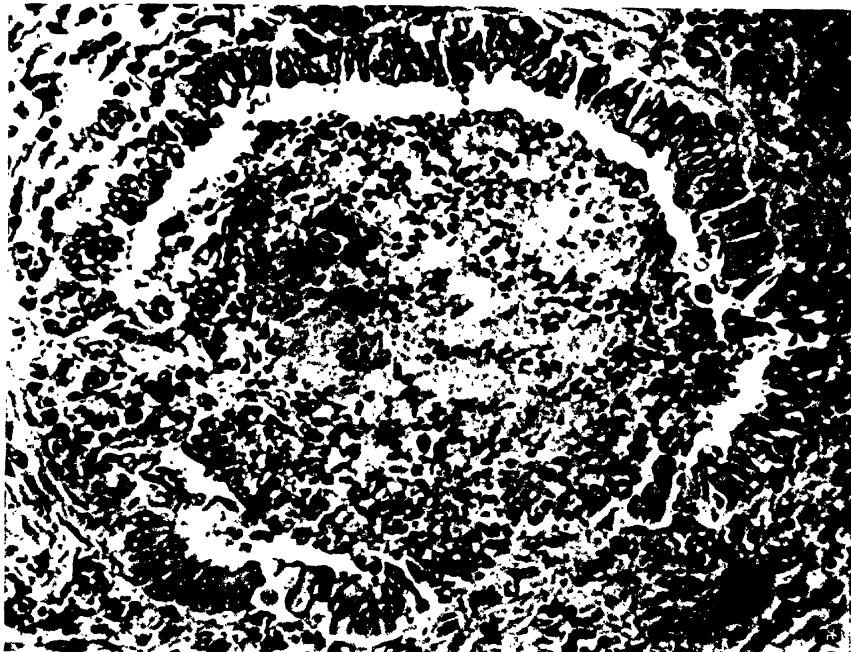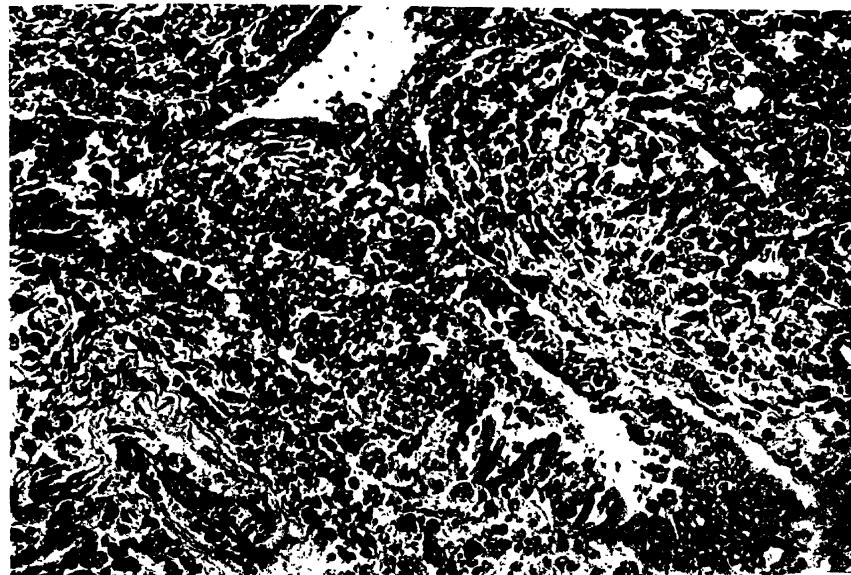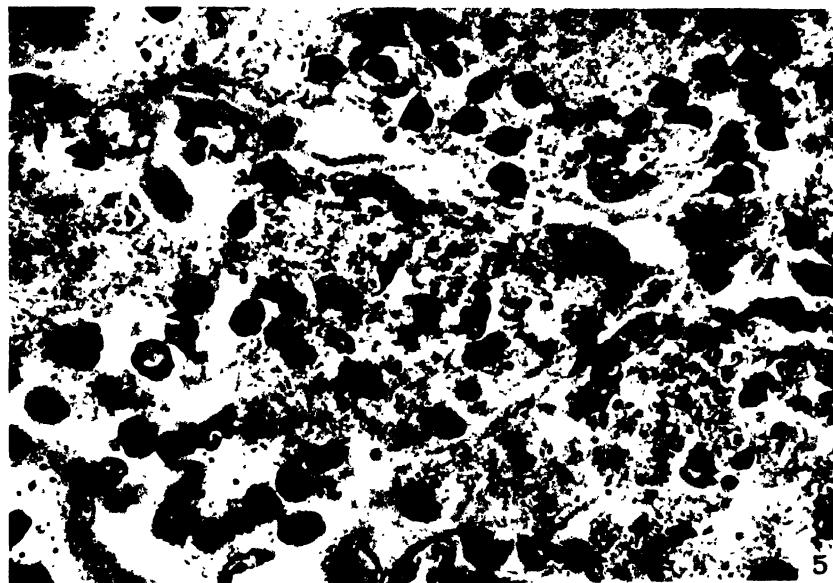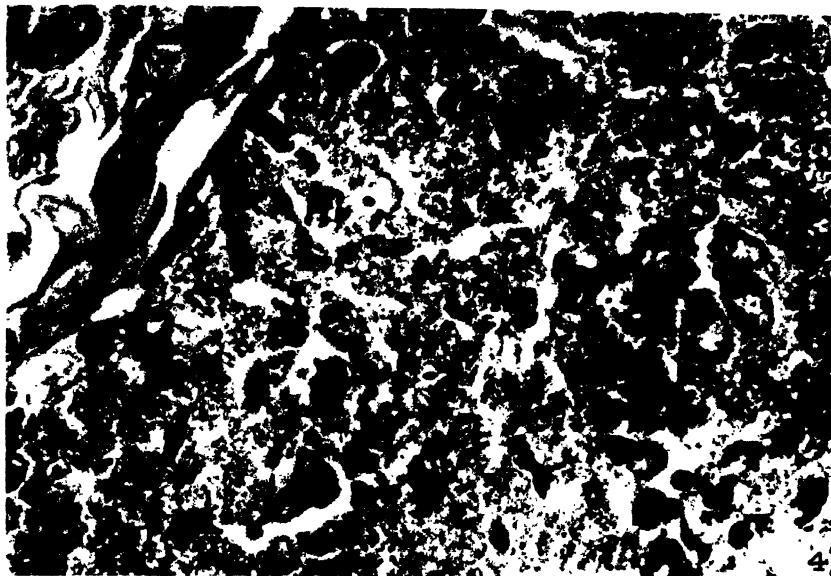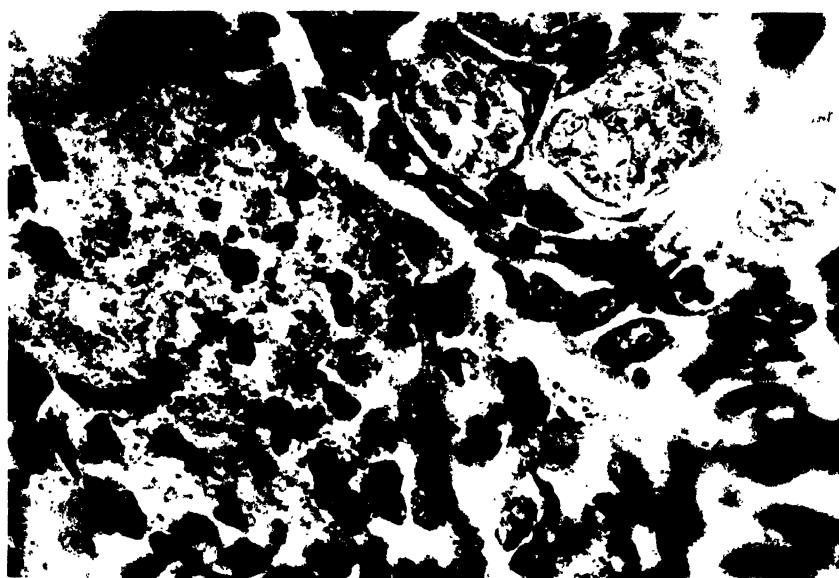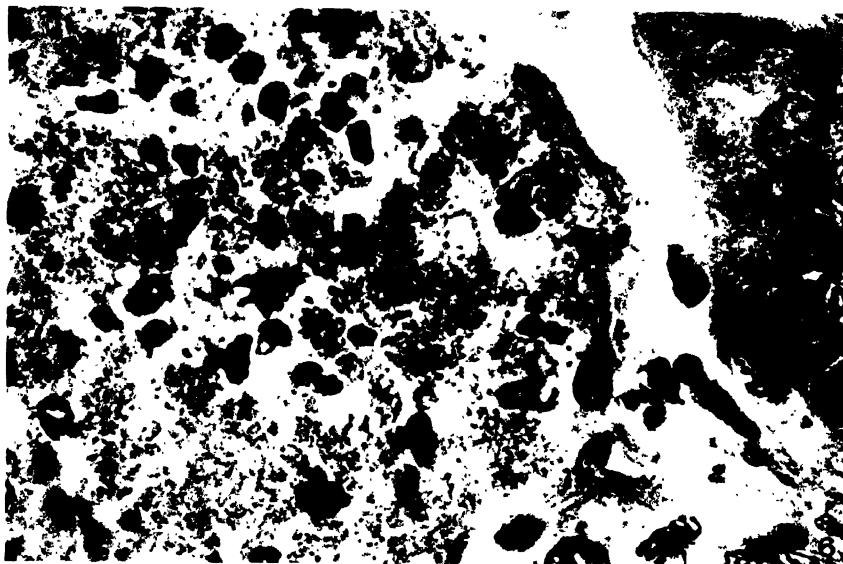
(Smith: *Bacillus actinoides.*)

(Smith: *Bacillus actinoides*.)

(Smith: *Bacillus actinoides.*)

10

11
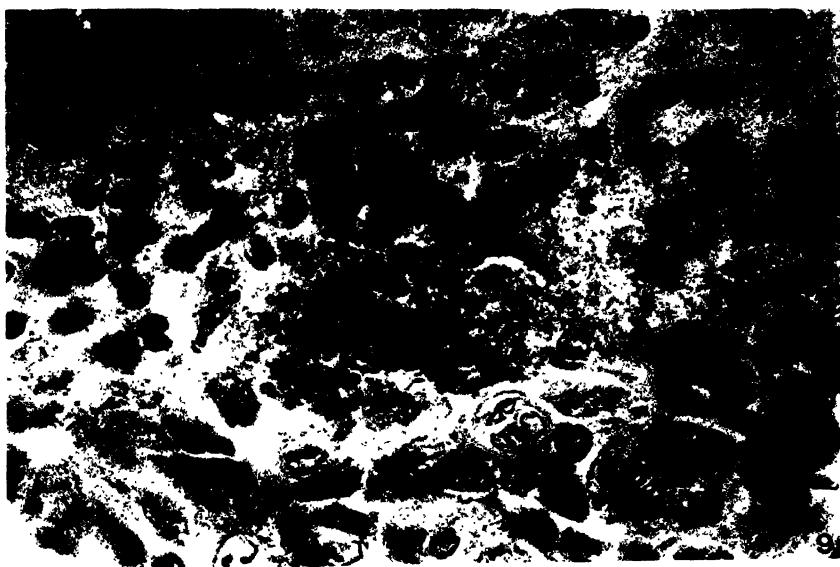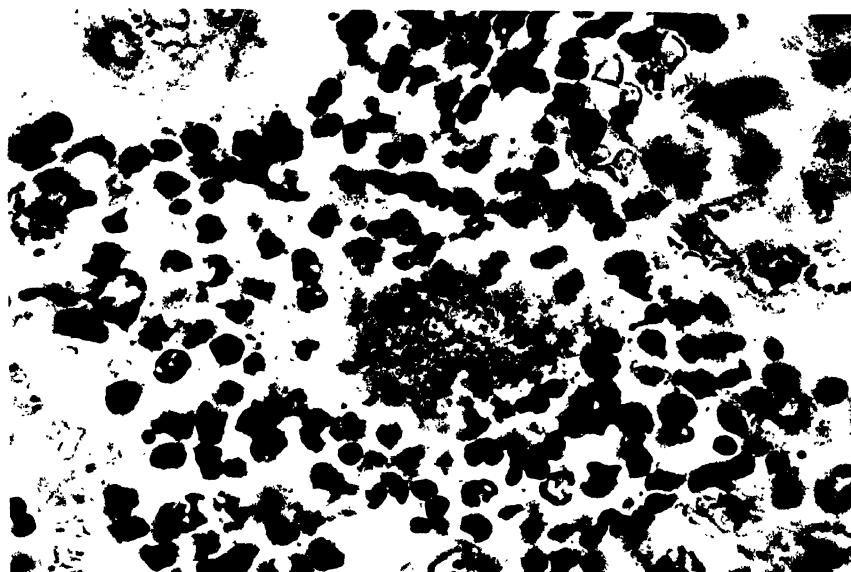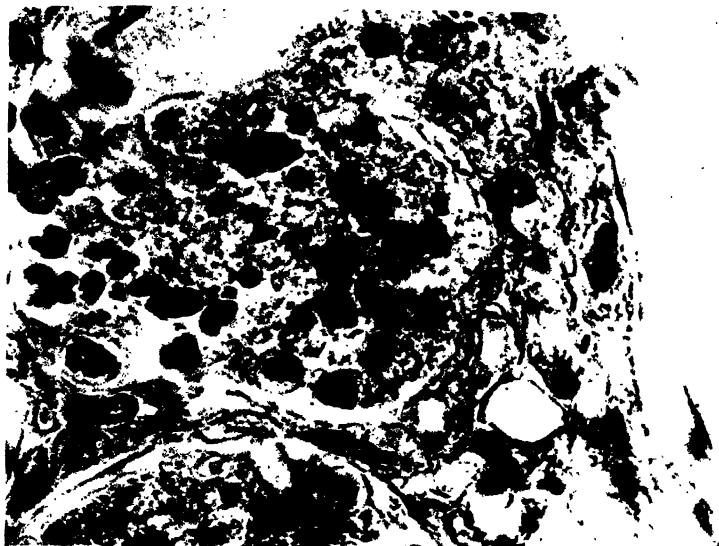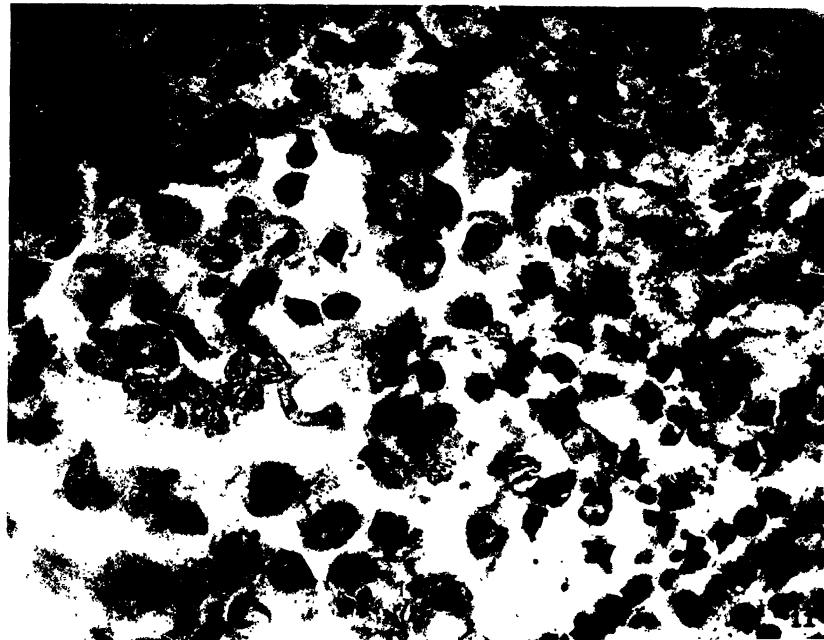
(Smith: *Bacillus actinoides.*)
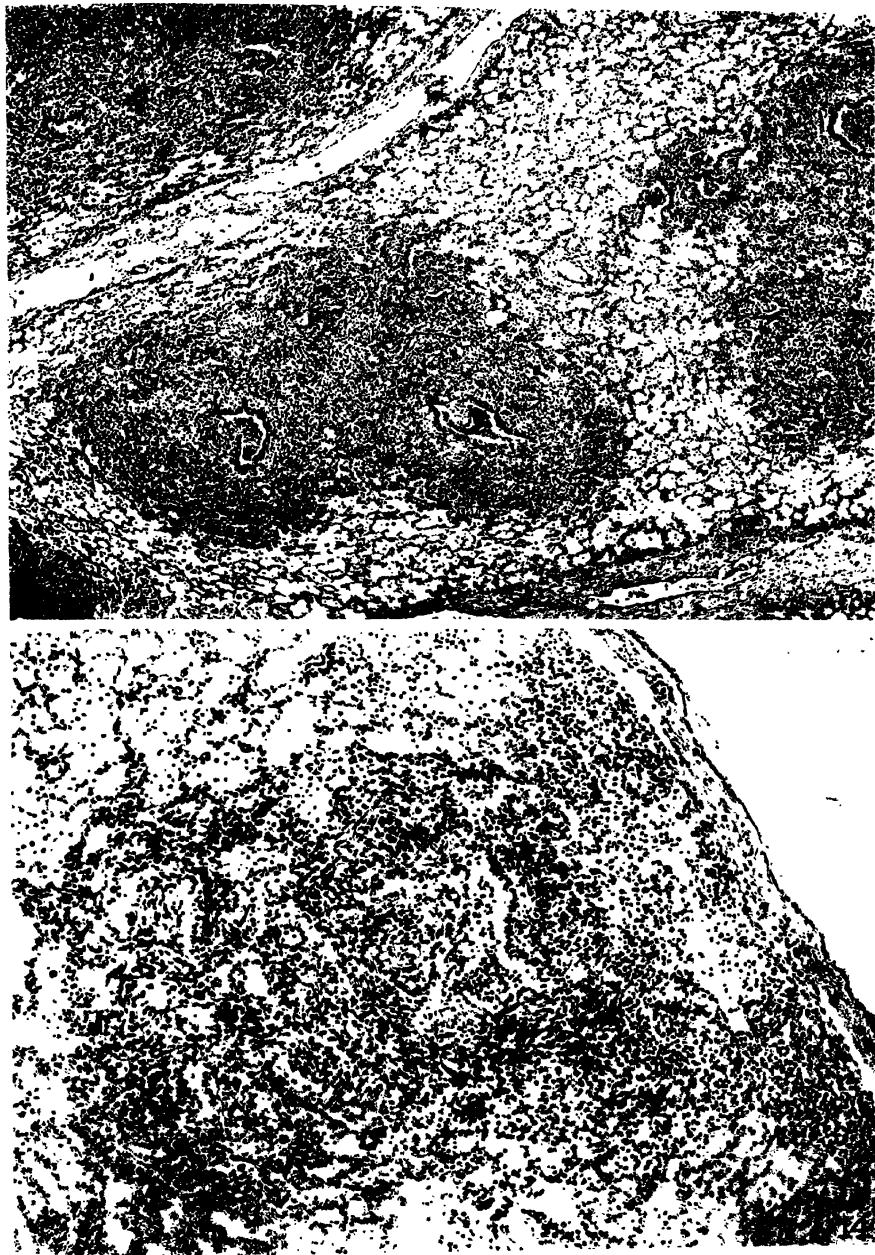
(Smith: *Bacillus actinoides.*)
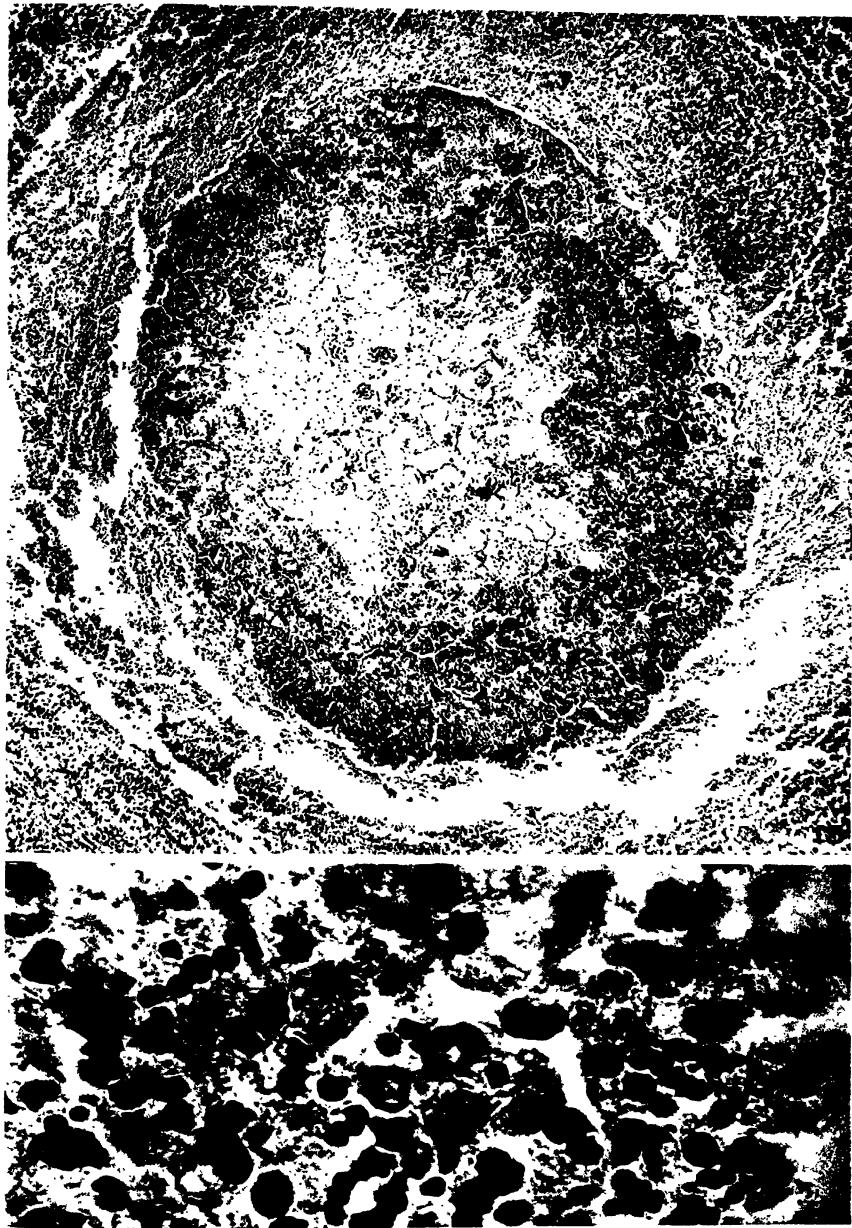
(Smith: *Bacillus actinoides.*)

THE INCIDENCE OF BLACKHEAD AND OCCURRENCE OF HETERAKIS PAPILLOSA IN A FLOCK OF ARTI-FICIALLY REARED TURKEYS.

By H. W. GRAYBILL, D.V.M.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

During the season of 1920 a flock of turkeys reared in incubator and brooder, and on new soil, has provided further data on the incidence of blackhead under such circumstances and has furnished additional evidence that this method is a successful means of rearing turkeys. Interesting data have also been obtained on the occurrence of the worm (*Heterakis papillosa*) shown in previous work to be a factor in the production of this disease and some evidence has been obtained on the source of this worm in the environment under which the experiments were conducted.

The ground on which the flock was reared was in a large, well fenced horse paddock which had not been used for turkeys or chickens for a period of more than 3 years. The soil was plowed and sown in the spring. The old turkeys and poultry were confined in enclosures at a distance from the paddock and precautions were taken to prevent attendants carrying infection from these to the flock in the paddock. As in the previous field experiments no attempt was made to exclude wild birds.

Three lots of eggs were incubated in commercial incubators. These yielded 85 poults. The lots were brooded separately and kept apart in coops and enclosures of their own until August 9 when they were combined into one flock and given the run of the paddock, within which the enclosures used up to this time had been located. Lot 1 was hatched May 30, Lot 2 June 9, and Lot 3 June 22. They were placed outdoors June 9 and 15, and July 1 respectively.

Up to October 1, nine had died of miscellaneous causes and four from blackhead. Twenty-eight in all had been removed at intervals

587

for experimental purposes. The remaining birds were in a flourishing condition. Of those that died of miscellaneous causes, one was trampled to death, in two the cause of death remained undetermined, two died of general weakness, two of leg weakness, one of typhlitis, and one of enteritis. After October 1, one other case of blackhead occurred and one more bird was removed for experimental purposes.

It will be noted that, in all, five cases of blackhead occurred in the flock. The dates on which the birds became sick were July 14, September 3, 15, 17, and October 5. The contents of one cecum in the first case were washed and sedimented for worms and one young *Heterakis papillosa* was found. The second case was not examined for worms. The contents of both ceca of the three remaining cases were washed and sedimented, and ten *Heterakis* small to full grown were found in one, one young and one full grown in another, and two full grown specimens in the third. Three of the birds were examined for coccidia with negative results.

With the exception of four birds retained for breeding, the remainder of the flock consisting of 42 birds was killed for food purposes during November and December. Data on these turkeys are given in Table I. The organs of all were examined for lesions of blackhead and for scars. The examination proved entirely negative. None of these birds had at any time shown symptoms of illness. All were normal at autopsy with one exception, in which the mucosa at the tips of the ceca was congested and pigmented.

The degree of infestation with *Heterakis* was determined by washing and sedimenting the cecal contents. It will be noted from the table that five were free of worms. Eleven turkeys harbored one worm each; eight, two worms; three, three worms; six, four worms; one, seven worms; three, eleven worms; and one, fourteen worms. As evidence of the difficulty the parasite has in establishing itself in a flock on virgin soil, it is of interest to note the number of cases in which either males alone or females alone were present. In twelve birds only males were present and in six only females. Further obstacles are presented to the multiplication of the parasite in that males alone presumably might occur in one cecum and females in the other, and moreover when both sexes occur together, individuals of both in the same stage of development might not be present, a circumstance not favorable to reproduction.

TABLE I.

Data on Turkeys Killed for Food.

No. of turkey.	Date killed.	Live weight.	Color.* Sex.*	No. of *H. papillosa* present.
	1920	*lbs.*		
430	Nov. 4	9½	B.	1 grown, 1 almost grown.
431	" 20		" M.	2 " females.
432	" 20	13¾	" "	None.
433	" 20	9½	W.; F.	"
434	" 20	8¼	B.; "	"
435	" 22	13¾	W.; M.	2 adult males.
436	" 22	13	" "	2 " females, 1 male.
437	" 22	12	" "	4 " " 6 nearly to full grown, 1 young male.
438	" 22	8	" F.	1 adult female, 3 young to grown males.
439	" 23	16½	B.; M.	1 young male.
440	" 23	13½	" "	3 grown females, 4 adult males.
441	" 23	14	" "	3 " males, 1 young female.
442	Dec. 18	13	" "	1 male nearly grown.
443	" 18	13½	" "	5 males and 5 females nearly to full grown, and a young stage 2–3 mm. long.
444	" 18	8¼	W.; F.	1 grown male.
445	" 18	8¼	B.; "	4 adult males.
446	" 18	9¼	" "	1 male and 1 female, adults.
447	" 20	15	W.; M.	1 grown male.
448	" 20	8¾	B.; F.	3 " females and 8 males nearly to full grown.
449	" 20	14¼	" M.	1 adult female.
450	" 20	14	" "	1 grown male.
451	" 20	16¼	W.; "	3 adult females and 1 male nearly grown.
426	" 21	9	B.; F.	2 grown females.
452	" 21	9¾	W.; "	1 " female, 1 adult male.
453	" 21	8	B.; "	14 males and females, nearly to full grown.
454	" 21	9½	" "	2 grown males.
455	" 22	10½	" "	1 nearly grown female.
456	" 22	10½	" "	1 " " male.
457	" 22	8½	" "	3 females nearly to full grown.
428	" 22	8½	" "	3 adult females, 1 male nearly grown.
458	" 22	10½	" "	1 grown female, 2 adult males.
459	" 23	12	W.; M.	1 " male.
460†	" 23	14¼	B.; "	None.
461	" 23	9¾	" F.	4 worms.
462	" 28	14	" M.	1 nearly grown male.
463	" 28	16	" "	2 " " males.
464	" 28	16¼	" "	None.
465	" 28	16½	" "	1 nearly grown female.

* B. indicates bronze; W., white; M., male; F., female.

† Mucosa of tips of ceca greatly congested and pigmented.

As evidence that the flock of turkeys was not one specially resistant to blackhead it will be of interest to consider the history of a number withdrawn during June and July and exposed to an old flock. The data on the incidence of blackhead and the occurrence of *Heterakis* in these furnish a striking contrast to what was observed in those not exposed.

Of twenty-nine birds removed for experimental purposes, sixteen were penned with the old flock beginning in July. All became

TABLE II.

Data on Turkeys Exposed to Old Flock.

No. of turkey.	Date exposed.	Date sick.	Blackhead.	Cecal contents washed and sedimented.	No. of *H. papillosa* present.
	1920	1920			
400	July 12	Aug. 2	+	Both ceca.	59 young to grown.
401	" 12	" 16	+	" "	21 "
402	" 12	" 8	+	" "	7 " to grown.
403	" 12	July 26	+	" "	About 25 young.
404	" 12	Aug. 16	*		
405	" 12	July 29	+	Both ceca.	11 young.
406	" 12	Aug. 8	+	" "	30 "
407	" 12	" 2	+	" "	90 "
408	" 19	" 6	+	†	
409	" 19	" 8	+	Both ceca.	100 young.
410	" 19	" 23	+	†	
411	" 19	" 16	*		
412	" 12	July 28	+	Both ceca.	7 young.
413	" 12	Aug. 8	+	" "	7 nearly grown.
414	" 12	" 23	+	" "	40 young to grown.
415	" 12	" 16	+	One cecum.	Many, mostly young

* Survived.

† No examination for worms.

sick within 42 days (Table II). Two survived and there is every reason to believe that they underwent an attack of blackhead, since both were sick for about 7 days. The diagnosis of blackhead in the others was confirmed at autopsy. In washing and sedimenting the contents of the ceca the number of *Heterakis* present was determined. In eight birds the number of worms ranged from 21 to 100. In the four remaining ones examined, the number found was 7 to 11. It should be stated that the method used in collecting the worms

would not lead to the detection of very young stages. Ten of the birds were examined at autopsy for coccidia with negative results.

Since the flock of normal turkeys was protected from infestation with *Heterakis papillosa* from other turkeys and chickens, the source of this parasite becomes a question for consideration. We have been fortunate in locating at least one of the sources of this infection in the ring-necked pheasant, present in this locality. In the previous fall a pheasant killed on a neighboring farm harbored specimens of this worm. Pheasants have been observed from time to time on the Institute farm and on a number of occasions they have been seen in close proximity to the laboratory and in the paddock in which this year's flock was reared. In June of this season attendants reported on a number of occasions a pair of pheasants present in this paddock. On November 18, a pheasant that had fallen a victim to hunters was found dead in the paddock and at autopsy specimens of *Heterakis papillosa* were found in the ceca. Considering the resistant character of the ova of this parasite, there seems little question that this bird, although present in small numbers, is capable of maintaining a certain degree of soil infestation with this parasite. Fortunately, this does not appear to be sufficiently concentrated to interfere with the successful rearing of turkeys when the soil has been subjected to ploughing and cultivation incident to planting. It will, however, be a safe practice to discourage the visits of this bird.

Experiment on the Persistence of the Ova of Heterakis in the Soil.

An enclosure that had proved infectious to turkeys during the season of 1919[1] remained occupied by recovered cases until January 21, 1920, at which time it became vacant and remained closed until June 28, a period of a little over 5 months. This covered a time of exceptionally severe winter weather with much ice and snow. On the latter date, four healthy turkeys, 29 days old, from the normal flock referred to above were placed in the enclosure. These turkeys contracted blackhead in 11, 21, 23, and 28 days respectively. *Heterakis* was present in all. The contents of both ceca were washed and

[1] Smith, T., and Graybill, H. W., *J. Exp. Med.*, 1920, xxxi, 633, Experiment 12, *b*

sedimented in the case of three of the birds. In one, seven worms were collected, and in the remaining two, many were found. Only one cecum of the fourth bird was examined and twenty-seven young *Heterakis* were collected.

Although the enclosure during the time it was unoccupied remained accessible to wild birds on the wing, the conditions for visits of hosts of *Heterakis* were on the whole unfavorable. Circumstantial evidence that the ova in the soil had survived and had not been introduced during the interval is furnished by the fact that the number of worms found per bird corresponded with that of the poults penned with the old flock and not with that of the flock on new ground, which was much more favorably located for the visits of birds.

SUMMARY.

In a flock of artificially reared turkeys originally consisting of 85 birds and reduced during the summer and fall by deaths and withdrawals for experimental purposes to 42 birds, five cases of blackhead occurred. These appeared during the months of July, September, and October. In four, *Heterakis* was searched for and found. In 38 birds from this flock killed for food during November and December, five harbored no *Heterakis*, and the rest carried light infestations.

Of sixteen healthy birds withdrawn from the above flock during July and placed with a flock of older birds which had passed through this disease in former seasons, all contracted blackhead and fourteen died of the disease. The infestation with *Heterakis* was, as a rule, high, reaching a hundred specimens in some cases. In general, it appears that a high infestation with *Heterakis* is correlated with a high incidence of blackhead, a relation that had already been inferred in feeding experiments. In both of these groups no other species of worm was found in the ceca, and in instances in which examinations for coccidia were made none was found.

Pheasants have been incriminated as a source of infestation with *Heterakis papillosa* in artificially reared flocks.

In an artificially reared flock 38 birds that had never been ill, when killed in November and December, failed to show lesions of blackhead or evidence in the nature of scars that they had passed through an attack of the disease.

Infectious soil that had remained unoccupied by turkeys and chickens for a period of 5 months beginning in the depth of a severe winter still harbored viable ova of *Heterakis* and proved highly dangerous to young poults.

These experiments and observations fail to throw any light on the source of the protozoan parasite (*Amœba meleagridis*) which causes the fatal lesions of blackhead.

AN IMPROVED ANAEROBE JAR.

BY J. HOWARD BROWN, PH.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

Laidlaw (1) introduced the use of platinized carbon as a catalyzer for the combustion of oxygen and hydrogen to produce conditions favorable to the growth of anaerobic bacteria. His method was adapted for use with single tube cultures only. McIntosh and Fildes (2, 3) utilized the principle for the growth of anaerobes in jars. Their catalyzer consists of a small amount of platinized or palladinized asbestos wrapped in a piece of wire gauze and suspended from the lid of the jar. Hydrogen is introduced into the jar through a stop-cock in the lid. The method of McIntosh and Fildes was modified by W. G. Smillie (4) who enclosed the platinized asbestos in a small perforated glass bulb attached to the end of the tube through which the hydrogen is introduced into the jar, the hydrogen thus entering the jar through the asbestos. Fildes (5) has published a very useful review of these and other methods for the growth of anaerobic bacteria.

In the above mentioned methods the catalyzer must be heated in a flame, the tube or jar quickly closed, and the hydrogen introduced before the catalyzer has cooled, or else combustion does not occur. When the reaction has once started, however, the heat of combustion is sufficient to carry it to completion. There may, however, remain in the recesses of the jar or tubes or within the media traces of oxygen which diffuse out into the jar very slowly after the catalyzer has cooled and combustion has ceased. It is to be noted that Smillie' sjar provided for intimate contact of the hydrogen but not of the oxygen with the catalyzer. He may have experienced some difficulty in this regard for in describing another form of apparatus he says: "The following method was devised to remove all the oxygen." The platinized asbestos was wrapped in a coil of fine nichrome wire the ends of which were joined to larger wires which passed upward through a rubber stopper. Hydrogen was introduced into a tube of inoculated solid medium and the rubber stopper inserted. The catalyzer was heated by passing an electric current through the coil surrounding it. Smillie points out that the tube may be set aside for a while and the asbestos reheated to ignite any residual oxygen. Apparently he got the most perfect anaerobic conditions with this apparatus. He did not utilize an electrically heated coil in jars in which many cultures could be enclosed, probably because of the danger of explosion when larger volumes of hydrogen and oxygen are used.
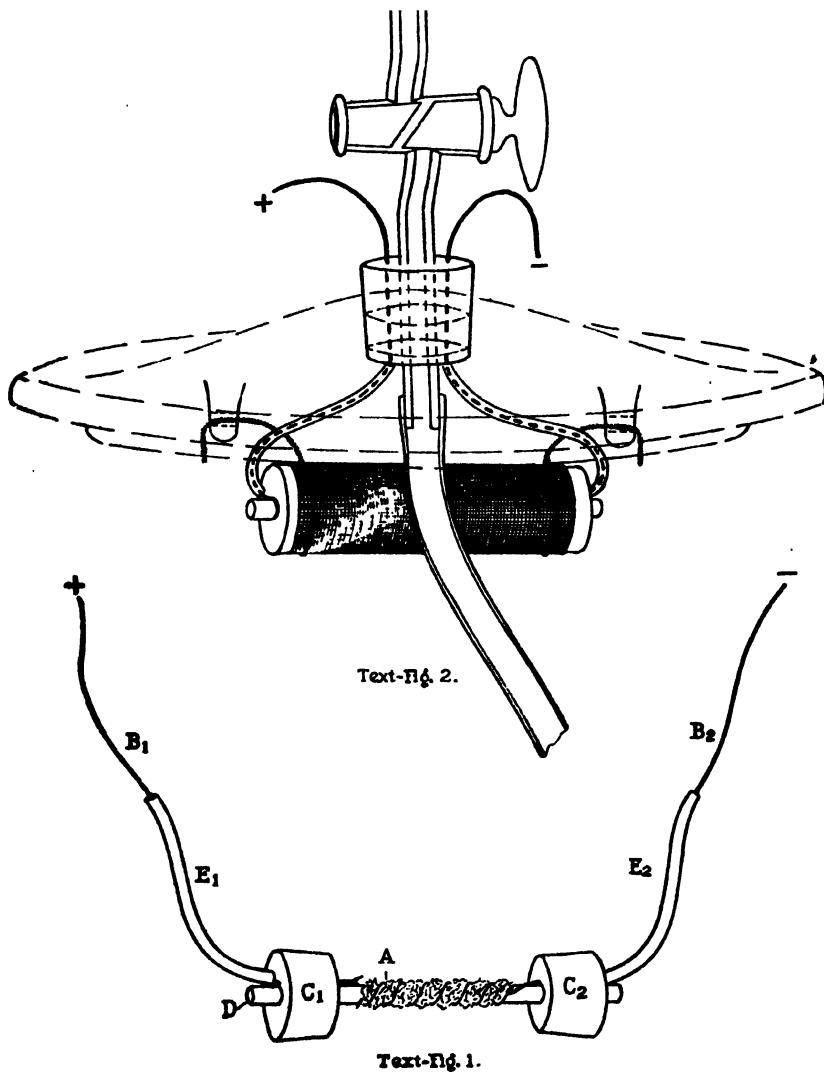
Text-Fig. 2.

Text-Fig. 1.

TEXT-FIG. 1. Showing the construction of the catalyzer coil. *A*, the fine nichrome wire coiled about the platinized or palladinized asbestos. *B*₁ and *B*₂, larger copper wires joined to the ends of the nichrome wire. *C*₁ and *C*₂, rubber stoppers. *D*, the core of glass tubing. *E*₁ and *E*₂, small rubber tubing serving as insulation.

TEXT-FIG. 2. Showing the coil enclosed by fine copper wire gauze and in position beneath the lid of the anaerobe jar.

596

In the apparatus to be described the danger of explosion is eliminated by completely enclosing the asbestos and coil of nichrome wire in a copper wire screen which does not come in contact with the coil or asbestos at any point. This introduces the principle of the Davy safety lamp. The apparatus is illustrated in Text-figs. 1 and 2, and is made as follows:

A piece of fine nichrome wire (B. and S. gauge No. 28) (Text-fig. 1, A) is joined at each end with pieces of larger copper wire (B_1 and B_2). One of the copper wires (B_1) is inserted through a No. 1 one-hole rubber stopper (C_1) and beside the wire is inserted also the end of a short piece of small glass tubing (D) holding the wire in the position shown in Text-fig. 1. Some palladinized asbestos is spread out onto a square of lens paper. This is then wrapped about the center of the glass tube and held in place by coiling the nichrome wire around it. The other copper wire (B_2) is then passed through another rubber stopper (C_2) which is placed over the other end of the glass tube. Pieces of small rubber tubing provide insulation for the copper wires at E_1 and E_2. A piece of fine copper wire gauze is rolled around the entire core and held in place by wires twisted about the stoppers at each end of the gauze. The twisted ends of these wires serve to fasten the cell to the lid of the jar as shown in Text-fig. 2. An ordinary round museum specimen jar is used. A one-hole rubber stopper carrying a glass stop-cock is inserted into a hole bored in the lid of the jar. The two copper wires coming from the coil are run up through the rubber stopper on either side of the stop-cock. This is easily done by sticking a large hypodermic needle down through the stopper, running the copper wire up through the bore of the needle and then withdrawing the needle, leaving the wire in place. In time leaks are likely to appear around the stopper, the wires, or the stop-cock, so that cement or sealing wax should be placed over and around the rubber stopper. From the lower end of the stop-cock a rubber tube leads to the bottom of the jar to insure a good mixture of the oxygen with the hydrogen entering the jar.

In use, the lid is clamped down onto the jar of cultures over a gasket of "plasticine" modeling clay. An electric current is connected with the two protruding copper wires and hydrogen is run into the jar through the stop-cock under pressure of about 5 pounds.

Combustion is soon manifested by the collection of moisture inside the jar and by the lid becoming quite warm. This is allowed to continue until the flow of hydrogen ceases, as may be detected by observing no more bubbles in the wash bottles of the hydrogen apparatus. 20 or 30 minutes are usually sufficient, after which the stop-cock is closed, the electric current disconnected, and the jar incubated. At any time during incubation the electric current may again be passed through the coil to consume any residual oxygen. Sufficient hydrogen for this purpose will remain in the jar. A tube of gelatin tinted with methylene blue and decolorized in boiling water just before being sealed within the jar serves as an indicator of the presence or absence of oxygen.

An electric light current of 110 volts reduced by passage through a 60 watt Mazda lamp has been used for heating the coil. Very little hydrogen is required since none is passed into and out of the jar as is the case with the Novy jar.

A number of coils and jars as described have been in use for several months. There have been no accidents due to explosion such as have been known to occur with other jars employing the combustion principle. Whenever perfect anaerobic conditions have not been attained the failure has been due to small leaks in the stopper or stop-cock. The coils have shown no deterioration with use or age. The bit of lens paper used to hold the asbestos in place when the nichrome wire is being coiled about it is burned off with the first passage of the electric current. Similar cells in which the hydrogen was introduced through the asbestos into the cell were made but found to possess no advantage over the one described.

SUMMARY.

There has been described a modification of the anaerobe jars of McIntosh and Fildes and of Smillie in which the oxygen is consumed by combustion with hydrogen under the catalytic action of platinized or palladinized asbestos.

The special advantages of the apparatus described reside in its greater safety and in the fact that the catalyzer is heated electrically after the jar is closed and may be reheated at any time during incubation without opening the jar.

BIBLIOGRAPHY.

1. Laidlaw, P. P., Some simple anaërobic methods, *Brit. Med. J.*, 1915, i, 497.
2. McIntosh, J., and Fildes, P., A new method for the isolation and cultivation of anaerobic microorganisms, *Lancet*, 1916, i, 768.
3. McIntosh, J., and Fildes, P., Nouvelle méthode d'isolement et de culture pour les microbes anaérobies, *Compt. rend. Soc. biol.*, 1916, lxxix, 293.
4. Smillie, W. G., New anaerobic methods, *J. Exp. Med.*, 1917, xxvi, 59.
5. Fildes, P., Methods for cultivating the anaerobic bacteria, *Med. Research Com., Nat. Health Insurance, Special Rep. Series, No. 12*, 1917, 59.

INDEX TO AUTHORS.

INDEX TO SUBJECTS.